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Effectiveness of *Piper betle* and *Cymbopogon citratus*, against *Vibrio parahaemolyticus*, Pathogen Caused Acute Hepatopancreatic Necrosis Disease (AHPND) on Whiteleg Shrimp, *Penaeus vannamei*

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Abstract: Acute Hepatopancreatic Necrosis Disease (AHPND) is a bacterial disease caused by *Vibrio parahaemolyticus* and has caused mortality from 40 to 100% of farmed marine shrimp. This study focused on shrimp, *Penaeus vannamei* survival fed with herbal diets before challenged with AHPND-causing bacteria *V. parahaemolyticus*. Screening antibacterial effects of betel leaves (*Piper betle)* and lemongrass (*Cymbopogon citratus*) against pathogen *V. parahaemolyticus* showed betel leaf having moderate positive antimicrobial activity while lemongrass presented weaker antimicrobial activity. Three groups of specific pathogen free shrimps, namely group A (normal pellet), B (normal pellet mixed with betel leaves) and group C (normal pellet mixed with lemongrass) were fed for 14 days prior to challenge test with *V. parahaemolyticus* suspension of bacterial density 1 x 108cells/ml. Results showed a higher survival rate, 87.5% in shrimp receiving betel leaves extract compared to 37.5% in group received lemongrass. A negative and positive control group had survival rates of 100% and 50%, respectively. After 24 hours of observation, all groups except the negative control had positive AHPND lesions and bacterial *V. parahaemolyticus*. This study shows that betel leaf extract has good potential in the treatment of bacterial diseases in shrimp and that feeding shrimp with betel leaves extract has promising results for minimizing the occurrence of AHPND. Further research is needed to examine the effects of betel leaves extract at the farm level.

Keywords: AHPND/EMS, herbs extraction, challenge test, bacteria, white shrimp

Abstrak: Penyakit Nekrosis Hepatopankreatik Akut (AHPND) ialah penyakit bakteria yang disebabkan oleh *Vibrio parahaemolyticus* dan telah menyebabkan kematian daripada 40 hingga 100% pada udang marin yang diternak. Kajian ini tertumpu kepada kemandirian udang, *Penaeus vannamei* yang diberi makan dengan diet herba sebelum dicabar dengan bakteria penyebab AHPND-*V. paraemolyticus*. Saringan kesan antibakteria daun sirih (*Piper betle*) dan serai (*Cymbopogon citratus*) terhadap patogen *V. parahaemolyticus* menunjukkan daun sirih mempunyai aktiviti antimikrob positif sederhana manakala aktiviti antimikrobial yang lebih lemah untuk serai. Tiga kumpulan udang bebas patogen spesifik, iaitu kumpulan A (pellet biasa), B (pellet biasa dicampur dengan daun sirih) dan kumpulan C (pellet biasa dicampur dengan serai) diberi makan selama 14 hari sebelum ujian cabaran dengan suspensi 1 x 108sel/ml bakteria *V. parahaemolyticus.* Keputusan menunjukkan kadar kemandirian yang lebih tinggi, 87.5% dalam udang yang menerima ekstrak daun sirih berbanding 37.5% dalam

kumpulan menerima serai. Kumpulan kawalan negatif dan positif masing-masing mempunyai kadar kemandirian 100% dan 50%. Selepas 24 jam pemerhatian, semua kumpulan kecuali kawalan negatif mempunyai lesi AHPND positif dan bakteria *V. parahaemolyticus*. Kajian ini menunjukkan bahawa ekstrak daun sirih mempunyai potensi yang baik dalam merawat penyakit bakteria pada udang dan pemberian makanan dengan ekstrak daun sirih berpotensi untuk meminimumkan kejadian AHPND. Kajian lanjut diperlukan untuk mengkaji kesan ekstrak daun sirih di peringkat ladang.

Introduction

Acute Hepatopancreatic Necrosis Disease (AHPND) of shrimp is a type of disease caused by a strain of *Vibrio parahaemolyticus* that is bound to release potent toxins PirAvp/PirBvp (Han et al., 2015). Accordingly, the toxins cause tissue loss and hepatopancreatic dysfunction, which result in mortality (Zorriehzahra and Banaederakhshan, 2015). AHPND often happens 35 days or less after postlarvae (PL) are stocked in ponds. Extremely high mortality rates were observed in infected shrimp ponds in their early stage of growth cycles. AHPND was reported in China, Vietnam, Malaysia, Thailand, Mexico, and the Philippine in 2009, 2010, 2011, 2012, 2013 and 2014 respectively (Tran et al., 2013; Joshi et al., 2014; Soto-Rodriguez et al., 2015; Dabu et al., 2015; Kua et al., 2016). Losses due to EMS/AHNPD was reported in Peninsular Malaysia, with estimation losses based on production at RM1.6 billion (USD 0.49 billion) between 2011 to 2013 (Kua et al., 2018).

Since the first occurrence of AHPND in 2009, there were numerous remedies solution have been put in practice by local shrimp farmers. Among them were replacing the culture species to fish, introducing multitrophic species, using garlic juice, and putting charcoal in the pond. In order to minimize losses due to disease outbreaks or mortality, shrimp farmers used various chemicals, including antibiotics. Use of antibiotics is permitted in Malaysia when prescribed by a competent authority and controlled under specific policies related legislations covering most of the antimicrobial used pathways (MOH, 2017). As well as included the management and control of antimicrobial in aquaculture under the National Fish Health Strategy 2018-2022 (DOF, 2018). The Poison Act, Feed Act, Fisheries Act, and Food Act are the primary acts that address AMR and AMU management in Malaysia. According to Defoirdt et al., (2011), antibiotics had been applied to shrimp farm production as a bacterial disease treatment. Unfortunately, prolonged and irresponsible usage of antibiotics in the shrimp industry can contribute to bacterial resistance and is not cost-efficient in the long term. Han et al., (2015) highlighted on antibiotic resistance detected on AHPND pathogenic bacterial strains in Vietnam. Due to this condition, an alternative approach, such as natural substance from herbs for the prevention of bacterial disease, should be emphasized.

Furthermore, the growing concern about the usage of antibiotics toward human food safety has led to the development of control foodborne pathogens by using antimicrobial compounds. Alzoreky et al., (2002) reported that antimicrobial compounds in herbs were found to possess antimicrobial activity. Natural substance from betel leaves (*Piper betle*) and lemongrass (*Cymbopogon citratus*) extracts are known to have antimicrobial activity against pathogenic bacteria in human (Liao et al., 1999; Sivaram et al., 2004; Bhattachary et al., 2007; Satish et al., 2008; Subashkumar et al., 2013).

In vitro studies on betel extract against pathogenic bacteria isolated from cultured fishes showed that the extract was able to inhibit the growth of *Vibrio alginolyticus*, *Vibrio vulnificus, Aeromonas hydrophila*, *Streptococcus* spp, *Photobacterium damsela* and *Micrococcus* sp. (Nik-Haiha et al., 2011). Similar results were obtained *in vitro* for Vibriosis caused by *Vibrio alginolyticus* in Asian seabass, *Lates calcarifer*, Motile Aeromonas Septicemia (MAS) caused by *Aeromonas hydrophila* in *Pangasius sutchii* and Nocardiosis in red snapper, *Lutjanus erythropterus* indicating the potential betel extract as alternative medication against bacterial diseases in fish (Nik-Haiha et al., 2014, Bond

& Senggagau, 2019 and Nik-Haiha et al., 2011). As for lemongrass extract, it was effective to be used as one of the therapeutic herbs against marine leech in hybrid grouper (Fadzilah & Azmi, 2018). Pathirana et al., (2019) demonstrated that lemongrass' oil possessed bactericidal activity against *Lactococcus garviae*, *Streptococcus iniae*, *Edwardsiella tarda* and *S. parauberis* isolated from olive flounders in Korea. Othman et al., (2018) suggested the best concentration of 100 mg/L of betel leaves crude extract as antimicrobial agent against marine bacteria.

Due to numerous reports on betel and lemongrass extracts on their antimicrobial properties, we carried out on the application of these two plant extracts in preventing or treating bacterial diseases in shrimps. The goal of the current study was to ascertain if betel leaves and lemongrass extracts could lower the prevalence of the *V. parahaemolyticus* bacteria causing AHPND in white shrimp.

Materials and Methods

Source of shrimps

Arca Biru Sdn Bhd in Kedah provided a total of 60 specified pathogen-free (SPF) white shrimps that had been in culture for 20 days. Using histopathology and an EMS-2 detection kit, the shrimps were evaluated and found to be AHPND-free. For the tests, only the prawn batches that tested negative were used. They were split into three groups (A, B, and C), with 30 white shrimps in group A (normal pellet), 15 in group B (normal pellet mixed with 100 ppm betel leaf), and another 15 in group C (normal pellet mixed with 200 ppm lemongrass). The shrimps were fed for 14 days, and then those in group A were further split into two groups, positive and negative control groups, in advance of the challenge tests.

Source of bacteria V. parahaemolyticus

Bacterial isolate identified as 3 HP, obtained from Shrimp-virus interaction laboratory (ASVI), CENTEX SHRIMP in Thailand was used for challenge test. Bacterial isolate was prepared from -80 °C glycerol-stock that was sub-cultured onto Trypticase Soy Agar medium supplemented with 1.5% NaCl. After overnight culture at 30 oC, 20 - 25 colonies of the bacteria were inoculated in Brain Heart Infusion broth and were shaken overnight at 30 oC to prepare the bacterial suspension for the challenge test.

Source of lemongrass and betel leaves

The products SitroPro® (PI 2017703131) and SirehMax® (MY-172900-A), which contain lemongrass and betel leaves, respectively, were both extracted in accordance with the prescribed procedures (FRI, 2022). Briefly, lemongrass was purchased from a local supplier in Johor and betel leaves were procured from Terengganu. Lemongrass was cleaned, chopped, dried at 40 °C for 12 - 24 hours and grinded using laboratory grinding machine. Approximately 200 g of powdered lemongrass was mixed with 1 L of ethanol (ratio 1: 5) and kept for 2 - 5 days at room temperature. The mixture was filtered through muslin cloth and the residues were adjusted to the required concentration with the extraction fluid for further extraction. An aliquot of extracted liquid was subjected to rotary evaporator for 3 - 4 h at 70 - 80 °C. The extraction liquids were stored at 4 °C in chiller for further usage. As for the betel leaves, the betel leaves were made into a powder using a blender after being dried for 3 to 7 days at room temperature (24 to 32 °C) in a shady area. A 100g of dried powdered betel leaves were immersed in 1 L ethanol (ratio 1:10) and kept for 2-5 days at room temperature. The extract obtained was then filtered, evaporated to dryness with a rotary evaporator and re-suspended in ethanol (90% concentration) to achieve a stock concentration of 100 mg/ml. The plants were stored in dark vials at 4 oC until further use. Both lemongrass and betel leaves used ethanol as type of solvents during the

extraction period.

Antibacterial assay

The antibacterial activity of the herbal extracts was qualitatively determined by disc diffusion method as previously reported by Bauer et al., (1966) and Anderson (1974). Briefly, the bacterial inoculum was spread evenly onto the surface of the Mueller Hinton agar plates and allowed to dry for 10 minutes. Sterile filter paper discs were impregnated with 100 µl of each extract (100 mg/ml) and later transferred onto the inoculated agar surface. Oxytetracycline, 30 µg/disc (Oxoid, UK) and Oxolinic acid, 2 ug/disc (Oxoid, UK) were used as positive control and the solvent (ethanol) as a negative control. Each extract was assayed in triplicate. All the plates were incubated at 37 oC for 24 h. The antibacterial activity was evaluated by measuring the zone expressed as millimeter (mm) of inhibition against test organism.

Preparation of herbal diets for experiment

The concentration used for betel leaves extract was 100 ppm while 200 ppm for lemongrass extract (Abdullah et al., 2018; Shaharah et al., 2022; Fadzilah et al., 2015 and Fadzilah et al., 2018). The extract was mixed with 50 ml of distilled water before being sprayed equally on 1 kg of pellet. For After 30 minutes of air-dry, the mixed pellets were packed into 2 g per packet and stored in chilled refrigerator. The shrimps were fed 5% of its bodyweight three times daily for 14 days.

Immersion challenge test

The challenge test was conducted by immersion of five shrimps for 1 minute in a 1 L aquaria tank containing *V. parahaemolyticus* bacterial density 1 x 108 cells/ml. After 1 minute, the shrimps were transferred into a 2 L aquaria tank contained 1 x 106 cells/ml bacterial suspension of and observed for the mortality within 24 hrs. The challenge test used in the present study was slightly modified from Tran et al., (2013) method.

Detection of AHPND by PCR

After 24 hours post infection, the hepatopancreas organ from alive shrimp from each group was dissected symmetrically into two parts; one for PCR while another for histology study. As for the PCR, the hepatopancreas were homogenized and inoculated on TSA plate for bacteria growth. The *V. parahaemolyticus* bacterial isolates from each group were then extracted for DNA using G-spinTM Total DNA Extraction Kit (iNtRON Biotechnology, Korea). The amount of template DNA in the 20 µl PCR reaction volume was in the range of 100-150 ng. AHPND primer set 1, (AP1) (Flegel & Lo, 2014) was used for the amplification of AHPND bacterial DNA fragments. The following are the sequences of the forward (F) and reverse (R) primer sets: AP1F, 5'- CCT TGG GTG TGC TTA GAG GAT G -3' and AP1R, 5'- GCA AAC TAT CGC. PCR was carried out using 0.2 ml microfuge tubes. The total volume reaction mixtures were 20.0 µl which contained 13.8 µl sterile distilled water, 2.0 µl 10 x PCR buffer without MgCl

2, 0.4 µl of 25 mM deoxyribonucleotide phosphates, 1. 2µl of 2 5mM MgCl

2, 0.2 µl of each primer, 0.2 µl of 500 units *Taq* DNA and 2µl template DNA. The cycling reactions were conducted for 5 min at 94°C, followed by 30 cycles of 30-sec denaturation at 94 °C, 30-sec annealing at 60 °C and 60-sec extension at 72 °C, plus a final 10 min extension at 72 °C (Eppendorf). Gel electrophoresis of 5 µL PCR products was done in 1.5% agarose gel at 400mA, 70V at 45 minutes. Stained gel was viewed under UV-illumination using Gel-DOC for band detection. The positive band for AHPND was confirmed at 700 bp.

Detection of AHPND by histology

The preparation of specimens for histology was done following Bell and Lightner (1988). The

shrimp from each group were injected with Davidson's fixative before fixing in Davidson's solution for 24 - 48 hours. After 24 - 48 hours in Davidson's solution, the specimens were immersed in 70% ethanol until the processing date. The specimens were processed by an automatic tissue processor (Leica ASP 300) and embedded in paraffin wax, which was then sectioned at 5 micrometers, stained with Haematoxylin and Eosin (H & E), and finally mounted with DPX before being examined under a compound microscope (Leica DM5000B) connected to a digital camera (Leica DFC 320) associated with computer software (Leica QWin). The pathology confirmation of AHPND was described as sloughing of hepatopancreatic tubule epithelial cells, prominent karyomegaly, and melanized granulomas (Lightner, 1996; Leano & Mohan, 2012).

Statistical Analysis

A One-Way ANOVA was performed to determine the significant differences for the survival of shrimp after challenged with bacteria *V. parahaemolyticus* that caused AHPND in the group received herbs diet and without. All statistical analyses were executed at the significant level of 0.05 using the statistical program SPSS Statistic, Version 20.

Results and Discussion

The antibacterial activity of the ethanolic extract of betel leaves exhibited moderate antimicrobial effects on test organism indicated by a moderate (10 - 14 mm) zone of clearance (Fig.1). While methanolic extract of lemongrass showed weak activity against bacteria. The inhibitor zone observed for betel leaves was similar with reference antibiotics (oxolinic acid) discs but less compared with antibiotic oxytetracycline (Fig.1). As for the lemongrass extract, the inhibitor zone was less than both antibiotics as well as betel leave extract. The antibacterial assay results showed that extraction from betel leaves has a moderate positive antibacterial activity toward *V. parahaemolyticus* while a weaker antimicrobial activity for lemongrass extract. The betel leaves extract having the antimicrobial activity showed in the present study was also reported by Veronica and Julian (2013). In their study, the crude extract of betel was able to inhibit *V. parahaemolyticus* ATCC 17802. Studied on the inhabitation activity demonstrated in betel leaves extract human pathogen were reported due to fatty acids and hydroxychavicol component (Ramji et al., 2002 and Bhattacharya et al., 2007). Both components were able to exhibit antibacterial activity by targeting the structure and function of bacterial cell walls and membranes (Subashkumar et al., (2013). The same authors also highlighted that crude extracts of betel leaves contain one or more of the phytochemical compounds (sterol, chavicol, and tannin) which have inhibitory effects on the bacteria.

In the present study, the lemongrass extract showed weaker antimicrobial activity. Suree and Pana (2013) reported that lemongrass extract was active against only 17 strains (7 - 11 mm) of the 25 strains in human pathogenic bacteria. Behboud et al., (2012) reported that antibacterial effect of lemongrass was most significant against Gram positive bacteria compared with Gram negative bacteria. In the present study, the challenge bacteria *V. parahaemolyticus* was a Gram negative bacterial and this may be the contributing factor to the weaker antibacterial activity.

The results of the *V. parahaemolyticus* after 24-hour challenge test showed a higher survival rate of 87.5% (average 3.5 ± 0.71) in shrimp given betel leaves extract compared to 50% (average 2.5 ± 0.00) in the positive control group and 37.5% (average 1.5 ± 0.71) in the lemongrass group (Table 1). No mortality was seen in negative control shrimp. One-way Anova analysis showed there was a significant difference ($p < 0.05$) in survival between the groups. Tukey HSD analysis showed that two groups of survival were observed; in which the first group consisted of shrimp from negative and those

fed with betel leaves extract while the second consisted of shrimps from positive and those received lemongrass extract. *V. parahaemolyticus* bacteria was detected in all the groups except in negative control group after 24 hours observation using AP1 primer. Sloughing of hepatopancreatic tubule epithelial cells, prominent karyomegaly, and melanized granulomas were observed under histological sections and validated as characteristics diagnosis for AHPND. Positive pathology AHPND was also seen in hepatopancreatic organ of all the groups except shrimp from negative control group (Fig.2). Both positives AHPND detection either from PCR detection or histopathology further confirmed the mortality in the present study was due to bacteria *V. parahaemolyticus.*

The higher survival rate seen in shrimp fed with betel leaves extract could indicate that they have a better defense system against bacteria *V. parahaemolyticus*. Nalina and Rahim (2007) showed that crude aqueous extract of *Piper betle* leaves showed antibacterial effect against *Streptococcus mutans* while Subashkumar et al., (2013) showed its antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. On the other hand, shrimp fed with lemongrass extracts did not show any significant results. Results of betel leaves and lemongrass extract in the present study were not able to be compared with other study due to unavailability of report on application of these herbs in shrimps. However, there are some reports on using others herbs extraction for prevention of bacterial infection in farmed shrimp. A better resistance against *V. harveyi* in shrimps fed with 25 mg/kg feed of turmeric extract was reported by Vanichkul et al., (2010). One of the limitations of this study is the small number of shrimps used in each replicate. This is because we want to make sure the shrimp have enough food and space to avoid cannibalism. We believe that, shrimp fed a diet containing betel leaves extract exhibit resistance to *V. parahaemolyticus*. The present study showed that *Piper betle* leaves extract has a good potential for prevention and treatment of bacterial diseases particularly in preventing AHPND outbreak in shrimp. Betel leaf has low toxicity and is safe for *P. vannamei*, Furthermore, it is friendly to the environment and it will help farmers to minimize losses due to AHPND.

Table 1: Average survival (%) of shrimp (14-Days oral dietary) after challenged with pathogenic*V.parahaemolyticus* that caused AHPND

Group	Average survival (%) of post challenge shrimp	
	0 hour	24 hours*
Control negative	100.0 ±	100.0 ± 0.0 a
Control positive	0.0 100.0	50.0 ± 0.0 b
Betel leaves extract	± 0.0	87.5 ± 17.7 a
Lemongrass extract	100.0 ±	37.5 ± 17.7 b
	0.0 100.0	
	± 0.0	

*Note: *One-way Anova test on differences between means of survival of shrimp between groups showed a significant difference ($p < 0.05$). Superscript indicates the post hoc group by Tukey HSD analysis.*

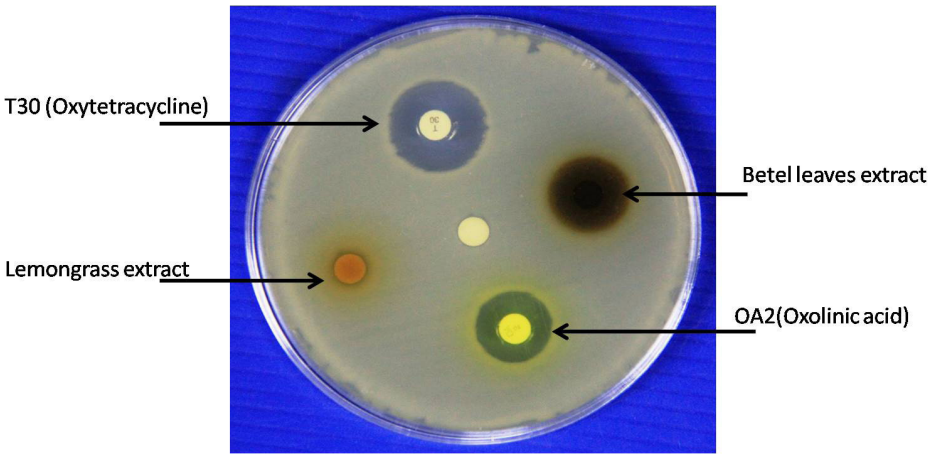

Figure 1: Results of antibacterial activity of betel leaves extract, lemongrass extract, oxolinic acid and oxytetracycline obtained using disc diffusion method of 24 hours *V. parahaemolyticus* grown cultures

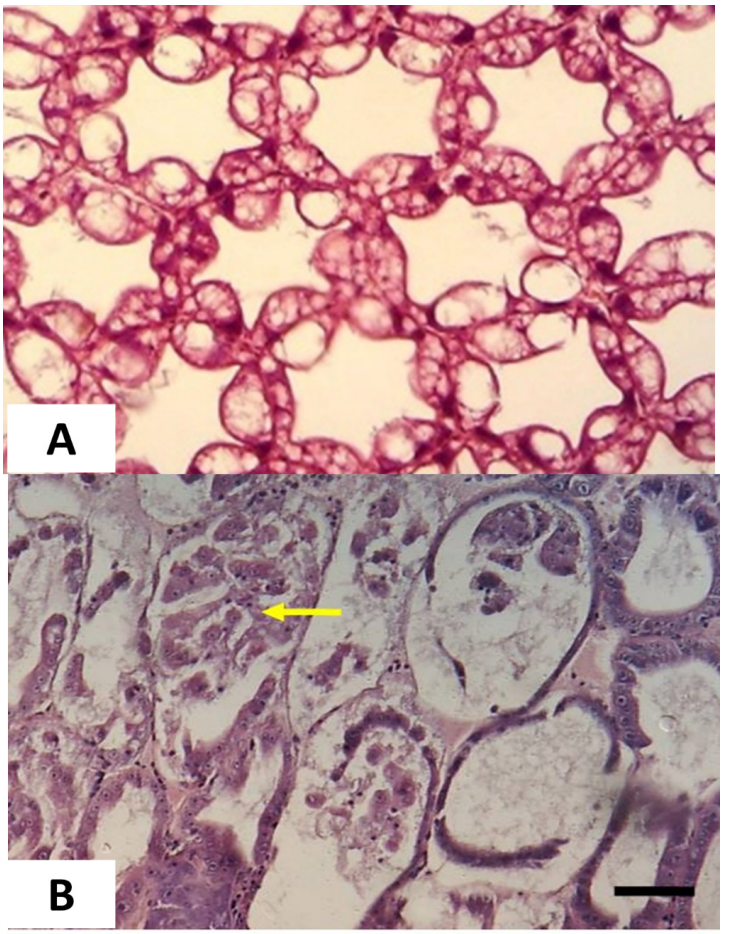

Figure 2: Histopathological finding from the hepatopancreas (HP) organ of post infection shrimps. No sloughing of epithelial cells from HP tubule was seen in negative shrimps (A) and sloughing of epithelial cells from HP tubule (arrow) were observed in all shrimps challenged with *V. parahaemolyticus*. H&E, Scale bars (A & B) = 50 µm

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First report of preparation of paralytic shellfish poisoning toxin standards from the toxic dinoflagellate *Alexandrium minutum* (Dinophyceae)

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Abstract: Paralytic Shellfish Poisoning (PSP) toxins are a group of potent neurotoxins that include saxitoxin and several structural analogues responsible for many poisoning incidents world- wide. PSP is the only harmful algal bloom (HAB) related shellfish poisoning that had been reported in Malaysia. A study has been conducted to purify the toxin compounds found in the batch cultures of *Alexandrium minutum* that is associated with PSP cases. A total of 15 batches of *A. minutum* algae cultures were extracted to determine the toxin levels using an isocratic, post-column derivatization HPLC method with fluorescence detection (HPLC-FLD). The major toxin groups detected in *A. minu- tum* cultures are from the GTX1&4, GTX5, and GTX2&3 groups. The highest amount of PSP toxin content detected was from batch AM07 at 7542.23 fg/cell. The culture was first purified through a Bio Gel P-2 resin to collect the fractions of the GTX1&4, GTX 5, and GTX2&3 toxin groups. The fractions of this toxin group were purified for the second time using Bio-Rex 70 resin to separate the toxin groups. Further study will be carried out to get the optimal amount of toxin. This is the first time to produce the toxin standard from the toxic dinoflagellate.

Keywords: HPLC-FLD, purification, PSP toxins, batch cultures, *Alexandrium minutum*

Abstrak: Toksin Keracunan Kerang-kerangan Paralitik (PSP) adalah sekumpulan neurotoksin kuat yang termasuk saxitoxin dan beberapa analog struktur yang menyebabkan banyak kejadian kera- cunan di seluruh dunia. PSP adalah satu-satunya keracunan kerang-kerangan berkaitan ledakan alga berbahaya (HAB) yang telah dilaporkan di Malaysia. Satu kajian telah dijalankan untuk menulenkan sebatian toksin yang terdapat dalam kultur kumpulan Alexandrium minutum yang dikaitkan dengan kes PSP. Sebanyak 15 kumpulan kultur alga A. minutum telah diekstrak untuk menentukan tahap tok- sin menggunakan kaedah HPLC terbitan pasca-lajur isokratik dengan pengesanan pendarfluor (HP- LC-FLD). Kumpulan toksin utama yang dikesan dalam kultur A. minutum adalah daripada kumpulan GTX1&4, GTX5 dan GTX2&3. Jumlah tertinggi kandungan toksin PSP yang dikesan adalah daripada kumpulan AM07 pada 7542.23 fg/sel. Kultur ini mula-mula ditulenkan melalui resin Bio Gel P-2 un- tuk mengumpul pecahan kumpulan toksin GTX1&4, GTX 5, dan GTX2&3. Pecahan kumpulan toksin ini telah ditulenkan buat kali kedua menggunakan resin Bio-Rex 70 untuk mengasingkan kumpulan toksin. Kajian lanjut akan dijalankan untuk mendapatkan jumlah toksin yang optimum. Ini merupakan kali pertama bagi menghasilkan piawaian toksin daripada dinoflagellat toksik.

Introduction

Marine biotoxins are chiefly produced by various types of toxic phytoplankton/microalgae. The proliferation of phytoplankton/microalgae producing marine biotoxins, also known as a harmful algal bloom (HAB), takes place globally. Approximately 300 species of marine microalgae are associated in harmful bloom events, of which over 100 species contain biotoxins that may cause human and animal poisoning or even death (Visciano et al., 2016). HAB has adverse environmental effects and, through bio magnifying the food web, can cause mass mortality to fish, birds, marine mammals, and human diseases by creating biotoxins that contaminate seafood (James et al., 2010; Visciano et al., 2016). The increased risk of shellfish toxicity to humans from HABs could result from large-scale ecological changes due to the anthropogenic activities, increased eutrophication, marine and aquacul- ture transport, and global climate change (James et al., 2010).

Several nations, including Malaysia, have reported HAB occurrences and accompanying shellfish poisoning. Paralytic shellfish poisoning (PSP), a HAB-related condition, is currently the most significant issue in this country. Dinoflagellates belonging to the genera Alexandrium, Gymnod- inium, Centrodinium, and Pyrodinium generate saxitoxin (STX), a strong neurotoxin that causes PSP (Harada et al., 1982; Negri et al., 2003; Murray et al., 2012; Shin et al., 2020).

The marine dinoflagellate species Pyrodinium bahamense var. compressum, Alexandrium minutum, Alexandrium tamiyavanichii, Alexandrium taylori, and Alexandrium peruvianum have all been identified as generating PSP toxin in Malaysian seas (Usup et al., 2002a; Lim et al., 2005). The first documented PSP occurrence in Malaysia occurred in 1976 on Sabah's west coast, where approxi- mately 201 people were poisoned including 7 of them passing away (Roy, 1977). This case was linked to Pyrodinium bahamense var. compressum (Böhm) Steidinger, Tester, and Taylor. Since that time, Sa- bah has experienced nearly yearly PSP incidents, and the Department of Fisheries Sabah has been reg- ularly monitoring HAB to ensure the safety of seafood (Jipanin et al., 2019). On the other hand, Pen- insular Malaysia's west and east coasts have experienced PSP since 1991. Three people were admitted to the hospital after consuming contaminated green mussels in Sebatu, Malacca in 1991 due to blooms of Alexandrium tamiyavanichii Balech (Usup et al., 2002a). One death was documented in a PSP case linked to the bloom of Alexandrium minutum located in Tumpat, Kelantan, in September 2001 (Lim and al., 2004), on which recurred in September 2015 (Lau et al., 2017). Due to the high concentration of saxitoxin in the clam tissue, the selling and collecting of shellfish from the region were outlawed (Borneo Post Online, 2015). Ten cases of PSP poisoning with the characteristic symptoms were re- corded from Kuantan, Pahang, in November 2013 and again in August 2014 (Mohammad-Noor et al., 20187). The oysters had been tainted with Alexandrium tamiyavanichii. The latest case of HABs that involved PSP toxic dinoflagellate, *Alexandrium minutum* was reported in the shellfish culture area of Sg. Geting, Tumpat, Kelantan in August 2020 (unpublished).

Due to natural poisons, the PSP is one of the worst seafood poisonings. Consuming bivalve mollusks that have been exposed to marine biotoxins, such as cockles, oysters, mussels, and clams, may cause severe intoxications (Nicolas et al., 2017). This biotoxin affects the mammalian nervous system by obstructing the sodium channel, which stops the neuron signal from being transmitted. According to Backer et al. (2003), high levels of PSP can result in serious sickness and mortality from respiratory arrest within a short period of time. By ingesting toxin-producing algae, both wild and farmed shellfish get contaminated with paralytic shellfish toxins, which have been linked to PSP in humans (Watanabe et al., 2011). PSP toxins may accumulate in filter-feeding species that consume di- noflagellates, such as molluscan shellfish, and may be transferred through the trophic chain (Deeds et al., 2008). The toxins are potentially fatal to humans or other consumers, including marine mammals and birds, although they do not appear to directly affect shellfish (Huang et al., 1996). Sabah's west coast has historically been the focus of phytoplankton observation in Malaysia, but the programme has

toxin compounds detected in the culture of *Alexandrium minutum* that is associated with PSP cases by several types of chromatography. HPLC then determined the purified toxins.

Materials and Methods

Chemicals and Standards

All of the solvents were HPLC-grade. Methanol, acetic acid, and acetonitrile of HPLC grade were purchased from J.T. Baker, Avantor, USA. Octanesulfonic acid and tetrahydrofuran were acquired from Fisher Chemical and Sigma, respectively, in the USA. Other substances were of an ana- lytical quality. An Ultra-Pure Water System (Evoqua Water Technologies, Germany) was utilised to clean the water for HPLC.

PSP Toxin Standards

Standard stock solutions of individual PSP toxins (Saxitoxin (STX), decarbamoylsaxitoxin (dcSTX) and gonyautoxin-5 (GTX5)) and standard stock solutions of mixed PSP toxins (gonyautoxin-1&4 (GTX1&4), gonyautoxin-2&3 (GTX2&3)) (NRC, Canada, Halifax) were purchased from the Groupe Biomedix Sdn Bhd. Selangor (except C-toxins, which were temporarily unavailable).

Culture of A. minutum

The pure culture in this study was obtained from Universiti Kebangsaan Malaysia (UKM), which was isolated during the PSP case in Geting, Tumpat, Kelantan in 2001. This species was confirmed to produce GTX4, GTX1 toxins up to 90 % of the toxin composition (Lim et al., 2007). Cultures were maintained in 10 L medium ES-DK (Kokinos and Anderson, 1995) at 15 ppt salinity, the temperature was maintained at 25 ^{o}C under a light intensity of 70 µmo photon/m^{2}/s below 16:8 h light:dark photocycle until the culture reaches the exponential growth phase (> 20,000 cells/mL). Phy- toplankton cell counts were performed using the Sedgwick Counter Chamber under the observation of the Inverted Microscope Olympus IX51 (Olympus, Japan).

PSP toxin extraction

The method of centrifugation was utilized to harvest the Alexandrium cultures for the toxin analysis (Eppendorf 5430, Hamburg, Germany). The cells were lysed using an ultrasonic homogenizer (OMNI-Ruptor 4000, Georgia, USA) following the process where the cell pellet was resuspended in 0.05 M acetic acid. The supernatant was then collected after the sample had been centrifuged at 10,000 g for 10 minutes. By running the extract through a 0.45 m nylon filter, the extract was cleaned thoroughly. Before further research, the extracted supernatant from the collected sample was kept at -20 °C. Toxin content was evaluated using HPLC.

PSP toxin analysis

The analysis of the toxins was executed using HPLC (Shimadzu, Japan) equipped with a Pickering post-column device and fluorescence detector utilising the isocratic, post-column derivat- ization with slight modifications method of Oshima (1995). The resulting samples were then separat- ed using a security guard cartridge (C18, 4 x 3.0 mm inner diameter) and a Luna C18(2) column (150 x 4.6 mm inner diameter, 120, 5 m) from Phenomenex in Torrance, USA, at a flow rate of 0.8 mL/min. The post-column temperature was set for roughly 65 °C for all runs while the column temperature was maintained at 27 °C. By substituting distilled water for the oxidising reagent, toxin verification was carried out in non-oxidizing post-column conditions. The reaction coil was kept in a cold bath during the analysis. The mobile phase for the STXs was 2 mM heptanesulfonate in a buffer solution of 30 mM ammonium phosphate and 5% acetonitrile (v/v), pH 7.1, and for the GTXs, it was 2 mM heptanesulfonate in a solution of 10 mM ammonium phosphate and 1% acetonitrile (v/v), pH 7.1. The post-column oxidising agent was 7 mM periodic acid in 10 mM sodium phosphate buffer, pH 9.0, and the acidifier was 0.5 M acetic acid. The sample injection volume for each run was 10 L, and the post column flow rate was 0.4 mL/min. Excitation and emission detection wavelengths were selected at 330 nm and 390 nm, respectively. Each sample was examined three times. Separate analyses of GTXs (GTX 1–5) and STXs (STX, dcSTX) were conducted. By comparing the identified toxin to standard

toxin materials, it was possible to identify and quantify the toxin. Each toxin's or epimeric pair's con- centrations (GTX1&4, GTX2&3, GTX5, STX, and dcSTX) were determined using linear calibration curves that were created using PSP-certified reference standards. The following values of the toxicity factor translate to STXequiv. were found for the calculation of toxicity from HPLC chromatograms: GTX1 (0.99), GTX2 (0.36), GTX3 (0.64), GTX4 (0.73), GTX5 (0.06), C1 (0.01), C2 (0.1), dcSTX (0.51), NEO (0.92), and STX (1).

Toxin purification

Accompanied with slight adjustments, the procedure outlined by Laycock et al. (1994) was used to discover how to purify GTXs from the sample extracts. A Bio-Gel P-2 column (fine; 25 mm x 200 mm; BioRad, Hercules, CA, USA) and a Bio-Rex 70 (20 - 50 mesh; 30 mm x 300 mm; BioRad, Hercules, CA, USA) were used to purify the extracts. These columns were equilibrated with deionized distilled water (Evoqua Water Technologies, Germany). The sample was loaded onto the column and eluted with 0.1 M AcOH at a flow rate of 10 mL/min. The Flash Chromatography Purification System (Teledyne ISCO, USA) was used for the collection of each ten-milliliter fraction. Further analysis and quantification of each fraction was done using HPLC. Fractions of each compound were combined, lyophilized, and then dissolved in 0.05 M AcOH. For further study, the samples were held frozen at -20 °C.

Statistical analysis

The statistical programme Statistical Package for Social Sciences (SPSS) version 16.0 for Windows was used to evaluate data on PSP concentrations. After performing a one-way analysis of variance (ANOVA) on the toxicity data to assess variations in the mean PSP of various batches of the A. minutum culture, Tukey's post hoc test analysis was performed. When P 0.05, means and standard deviations of values made in triplicate were reported and deemed substantially different.

Results and Discussion

In Malaysia, paralytic shellfish poisoning cases have been linked to many Alexandrium species (Usup et al., 2002b). HPLC toxin analysis showed that this sample's PSP toxins were created by A. minutum culture. The GTX group's chromatogram is displayed in Figure 1. A. minutum has been found to include the following primary toxin groups: GTX1, GTX2, GTX3, GTX4, and GTX5, with GTX1 and GTX4 being the dominating derivative (Figure 1a & b). In this order, the toxins were eluted: GTX4, GTX1, GTX5, GTX3, and GTX2. By comparing these toxins to the GTXs standard, it was determined that they belonged to the GTXs group (Figure 1c). Usup et al. (2006) also shown that only GTX1, GTX2, GTX3, and GTX4 were present in A. minutum. A. minutum only produced GTX 1 and 4, with GTX 4 being the dominant derivative, according to the majority of investigations con- ducted to date (Hwang and Lu, 2000; Yoshida et al., 2000; Lim et al., 2004). However, distinct toxin profiles have been discovered for some isolates, including neosaxitoxin (NEO) and sulfocarbamoyl toxins (C-toxins) produced by isolates from Denmark and New Zealand (Hansen et al., 2003) and New Zealand (Chang and McClean, 1997).

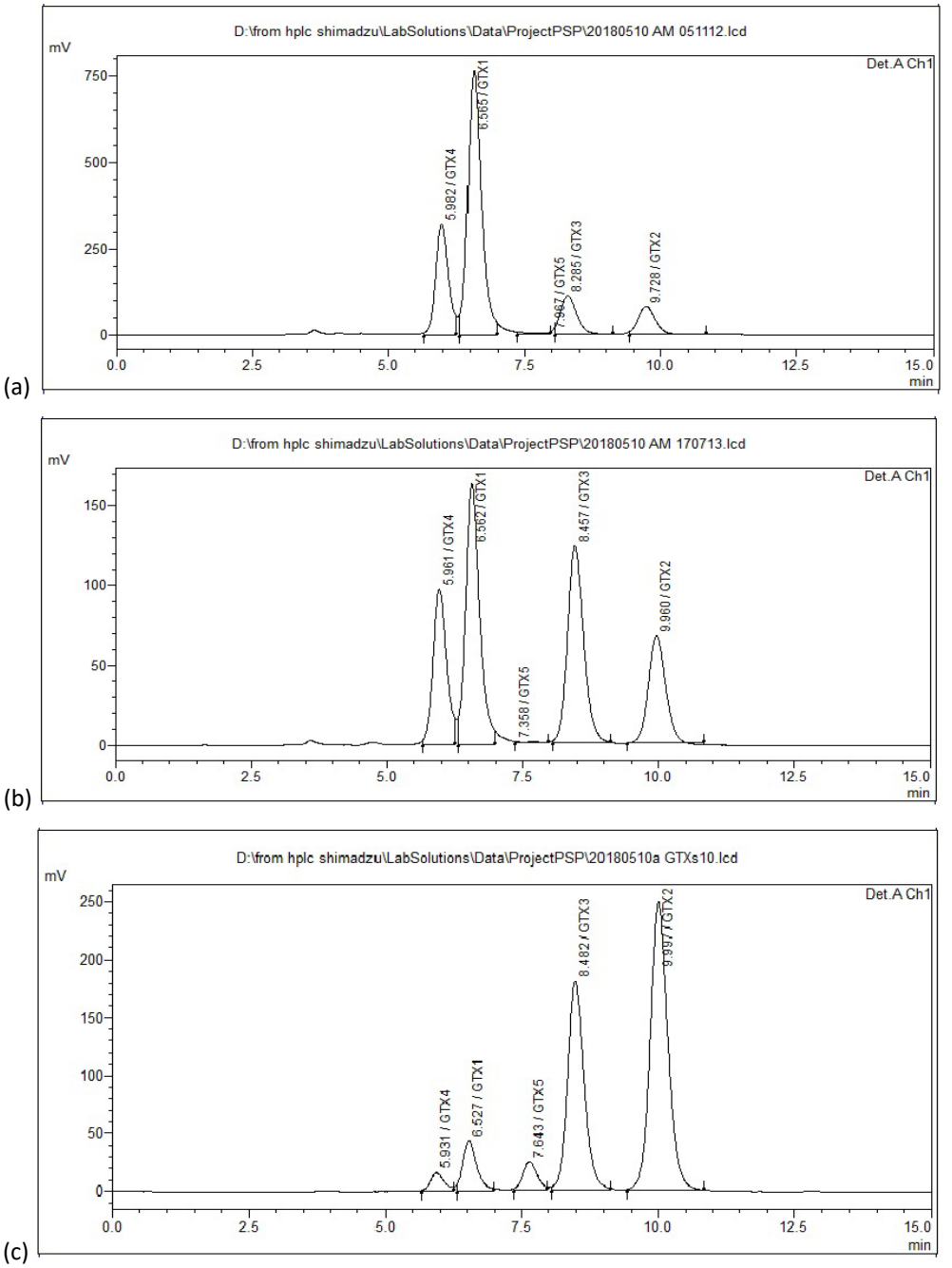

Figure 1. Toxin profiles of *A. minutum* from batch AM10 (a), batch AM09 (b) and GTXs standard (c).

A total of 15 batches of *A. minutum* cultures were extracted to determine the toxin levels in this study. There was a significant difference in mean total PSP (fg/cell) between the batches ($P < 0.05$). The highest cell toxin content was found from the batch AM07 at 7542.23 fg/cell followed by batch AM06 and batch AM08 with the mean total PSP of 4377.35 fg/cell and 4116.23 fg/cell, re- spectively (Figure 2). It has been shown that the toxin content of a cell varied widely depending on growth conditions (Usup et al., 1994; Cembella, 1998). Analysis showed that culture of *A. minutum*

from batch AM07 contained an average of 4569.79 fg/cell GTX4, 2733.74 fg/cell GTX1, 71.87 fg/cell GTX5, 110.03 fg/cell GTX3 and 56.79 f fg/cell GTX2. The other three toxins were absent in this culture (Figure 3).

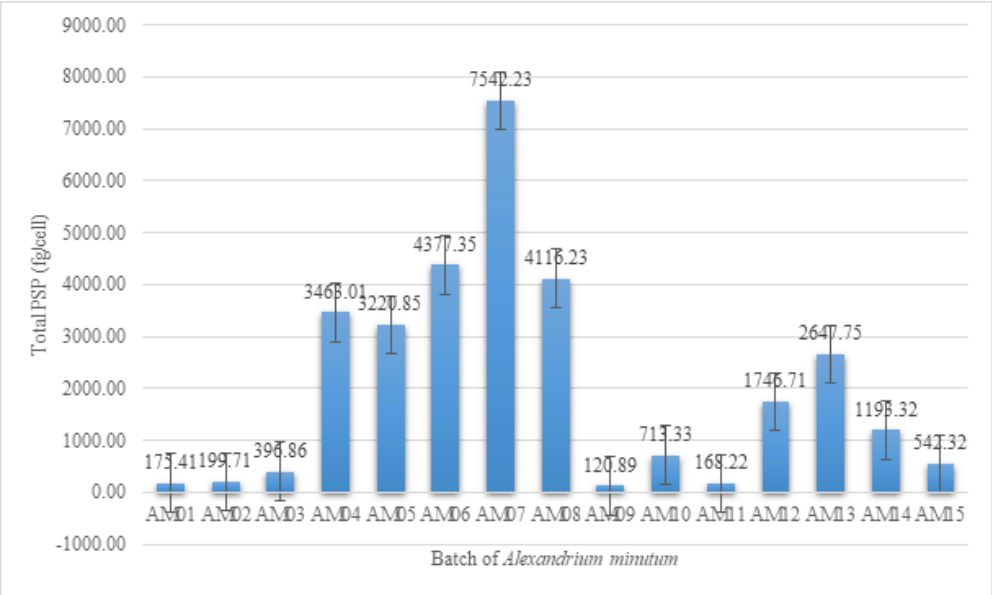

Figure 2. Mean of total PSP concentration (fg/cell) from different batch cultures of *A. minutum*.

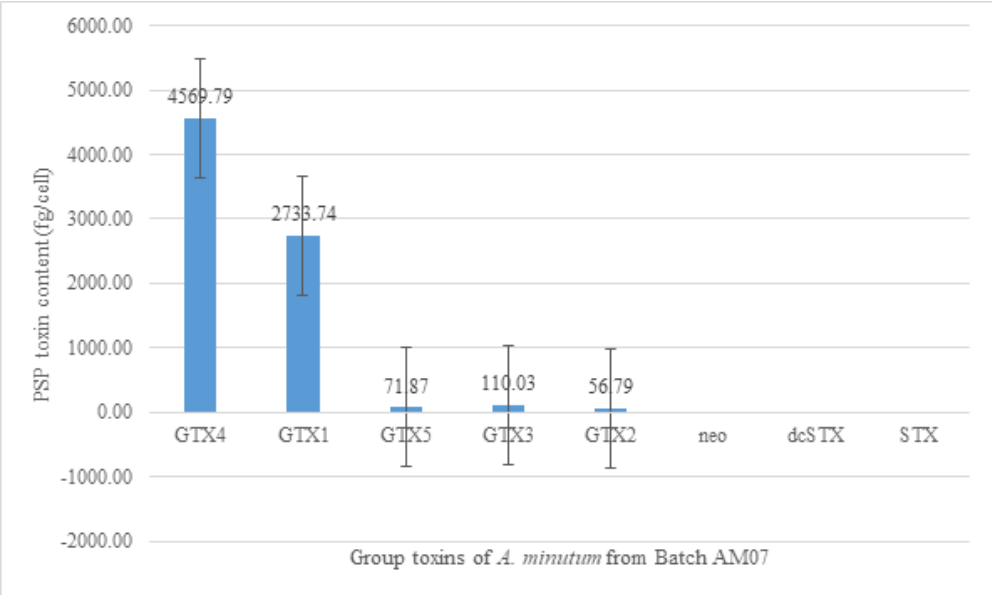

Figure 3. Mean of PSP toxins (fg/cell) in the culture of *A. minutum*.

The toxin obtained from each batch culture of *A. minutum* was subjected to Bio Gel P-2 column chromatography equilibrated with distilled water. The majority of other soluble small compounds in the extract can be effectively removed using this procedure. By using chromatography on Bio Gel P-2, it is necessary to carefully separate toxins with net charges of zero or negative integers. On the

column, the toxin was almost entirely absorbed. The toxin was then eluted with 0.03 M acetic acid after the column had been thoroughly cleaned with distilled water. Figure 4 displays an illustration of the elution profile from the batch culture of A. minutum.

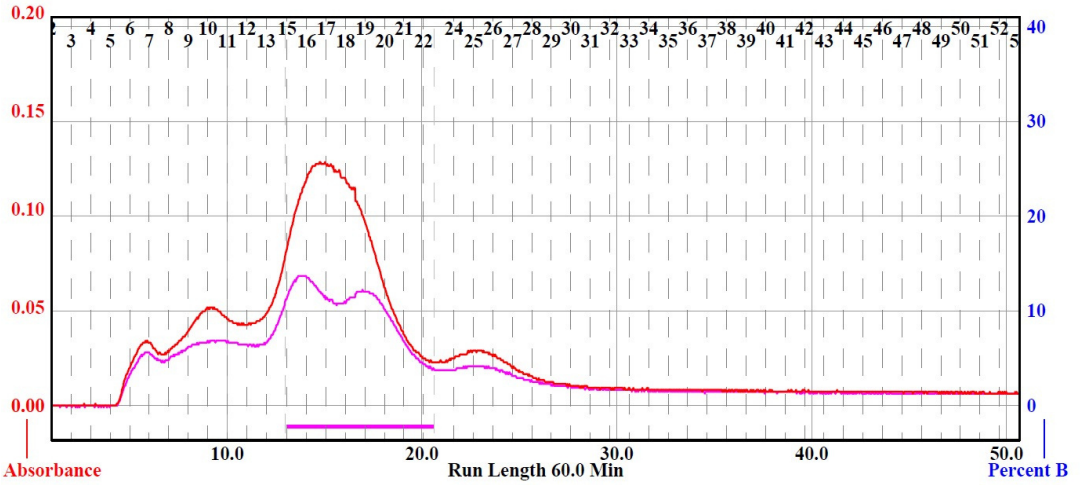

Figure 4. Elution profile of *A. minutum* culture from Bio Gel P2 column (2.5 x 20 cm)

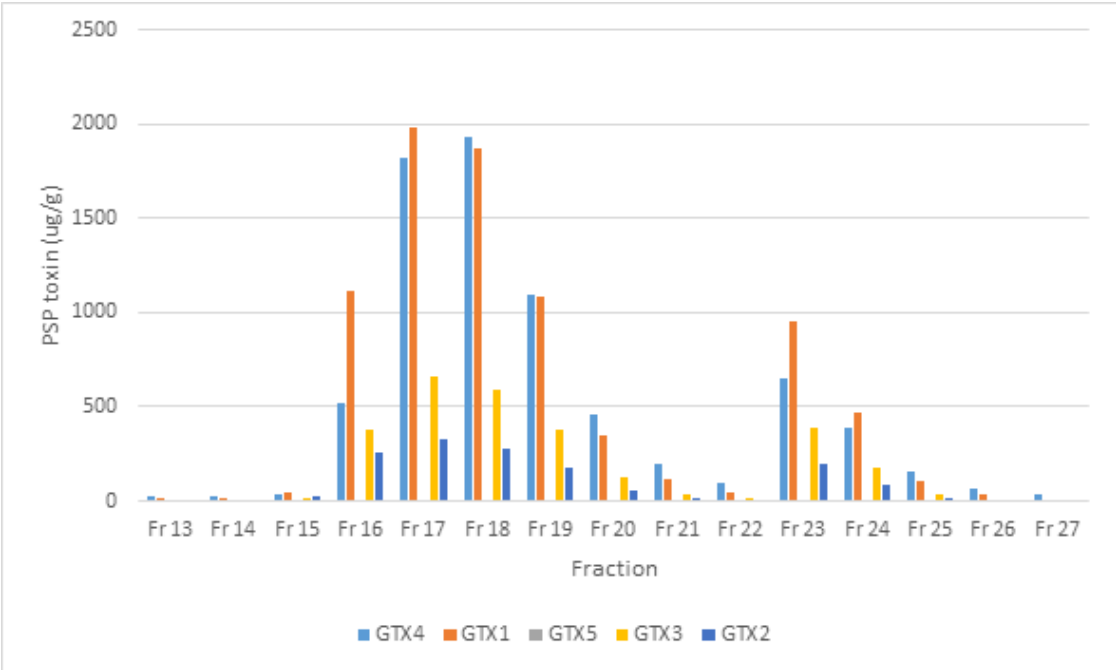

Figure 5. The content of PSP toxin groups from the batch of*A. minutum* culture after purification with Bio Gel P2 resin.

Tube from fraction 5 to fraction 28 were collected and analysed individually by HPLC. Analysis of the toxic fractions by HPLC showed that GTXs group was detected from fraction 16 to fraction 20 and fraction 23 to fraction 25. The range of total amount of GTXs in these fractions was 24.79 – 1931.18 µg/g (GTX4), 10.63 – 1986.85 µg/g (GTX1), 0 – 4.03 µg/g (GTX5), 2.73 – 659.24 µg/g

(GTX3) and 0.59 – 328.45 µg/g (GTX2). The toxic fractions were combined, concentrated and lyophilized. The toxins were further purified on a Bio-Rex 70 column with linear concentration gradients of acetic acid from zero to 3 M using 500 mL. However, no fraction was detected to contain GTXs group after purified by Bio-Rex 70 column. Figure 6 shows that the separation on Bio-Rex 70 was not complete for any of the five toxins. This might be due to the toxin was not adsorbed on cation-ex- change resin (Bio-Rex 70) because of high concentration of acetic acid or the column was not packed properly. Several alternative adsorptions were tried without significant improvement in resolving the toxins. For the Bio Gel P-2 column, resolution improved with column length and small sample vol- umes (Laycock et al., 1994).

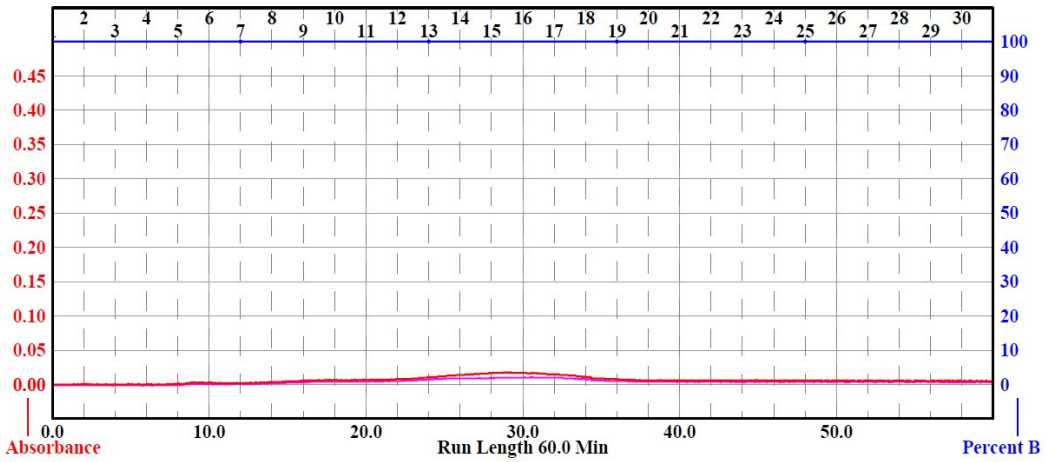

Figure 6. Elution profile of *A. minutum* culture from Bio-Rex 70 column (2.5 x 20 cm)

Conclusion

In the present study conducted, the main toxin groups identified in the current study's cultures of A. minutum were GTX1, GTX2, GTX3, GTX4, and GTX5, with GTX1 and GTX4 producing the dominating derivative. The results showed that the purified toxin from the *A. minutum* contained 5.56 µg/g (GTX1), 27.41 µg/g (GTX4), 1441.59 µg/g (GTX5), 1025.72 µg/g (GTX2) and 42.13 µg/g (GTX1) after purification by Bio Gel P-2 column chromatography. Nevertheless, further purification on Bio-Rex 70 was not accomplished for any of the five toxins. Further study will be carried out to gain the optimal condition on Bio-Rex 70.

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Microphytoplankton Composition and Water Quality Parameters of Mariculture Area in Kuala Gula, Perak, The Northern Straits of Malacca

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Abstract: Composition and abundance of microphytoplankton with an emphasis of potential harmful microalgae were monitored monthly in the marine fish cages area of Kuala Gula, Perak from February 2017 to January 2018 at six selected stations. Physical and chemical parameters of the sub- surface seawaters were also investigated. Microphytoplankton communities in the area consists of 64 genera, with 45 diatoms, 17 dinoflagellates and 2 blue-green algae. The microphytoplankton abundance was between 2.73×10^3 cells L-1 and 1.5×10^6 cells L-1. Diatoms dominated the microphytoplankton community with the relative abundance of 99.65 %. Diatoms, *Skeletonema* spp. dominated the sampling area more than 73.0 % throughout the sampling period with the highest percentage of contribution of 97.93 %. Potentially harmful microphytoplankton species were identified such as *Alexandrium* spp., *Chaetoceros* spp., *Dinophysis* spp., *Prorocentrum* spp., *Karlodinium* spp., *Margalefidinium* spp., *Noctiluca scintillans, Pseudo-nitzschia* spp., *Skeletonema* spp., and *Tripos* spp. No harmful algal blooms (HAB) occurred throughout the study period. However, a recent huge fish kills linked with massive bloom of *Margalefidinium fulvencens* was reported in May 2020 in this area. Hence, there is an urgent need to do continuous HAB monitoring and early warning program in this area to minimize the losses and safeguard aquaculture industries in future.

Abstrak: Komposisi dan kepadatan mikrophytoplankton telah dipantau setiap bulan di kawasan sangkar ikan marin Kuala Gula, Perak dari Februari 2017 sehingga Januari 2018 di enam stesen terpilih dengan penekanan kepada mikroalga berpotensi berbahaya. Parameter fizikal dan kimia permukaan air laut juga disiasat. Komuniti mikrofitoplankton di kawasan itu terdiri daripada 64 genera, dengan 45 diatom, 17 dinoflagelat dan 2 alga biru-hijau. Kelimpahan microfitoplankton adalah antara 2.73×10^3 cells L-1 dan 1.5×10^6 cells L-1. Diatom mendominasi komuniti mikrophytoplankton dengan kelimpahan relatif sebanyak 99.65 %. Diatom, *Skeletonema* spp. mendominasi kawasan persampelan melebihi sebanyak 73.0 % sepanjang tempoh persampelan dengan peratusan sumbangan tertinggi sebanyak 97.93%. Spesies mikrophytoplankton yang berpotensi berbahaya telah dikenalpasti seperti *Alexandrium* spp., *Chaetoceros* spp., *Dinophysis* spp., *Prorocentrum* spp., *Karlodinium* spp., *Margalefidinium* spp., *Noctiluca scintillans*, *Pseudo-nitzschia* spp., *Skeletonema* spp. dan *Tripos* sp. Tiada cetusan alga berbahaya (CAB) berlaku sepanjang tempoh kajian dijalankan. Walau bagaimanapun, laporan terbaru kematian ikan secara besar-besaran di kawasan ini telah dikaitkan dengan cetusan alga, *Margalefidinium fulvencens* yang dilaporkan pada Mei 2020. Oleh itu, terdapat keperluan mendesak untuk melaksanakan pemantauan CAB berterusan dan program amaran awal di kawasan ini untuk meminimumkan kerugian dan melindungi industri akuakultur pada masa hadapan.

Keywords: finfish mariculture, harmful algal blooms, fish killer, microalgae, water quality, Straits of Malacca

Introduction

The state of Perak is one of the major contributors to the production of farmed finfish in Malaysia. Intensive finfish culture areas are located in Kerian District and Larut Matang District, Perak. In the year 2018, Perak produced 5732.2 tonnes of marine fish valued at RM 119.61 million, contributing about 15.31 % of the total marine fish cages production in Malaysia (Annual Fisheries Statistics, 2018).

Production of cultured fish in Perak increased in 2019 stood at 6,948.17 tonnes valued at RM 190.06 million (Annual Fisheries Statistics, 2019). A total of 5,153.69 tonnes (74.1 %) out of 6,948.17 tonnes production of cultured marine fish in Perak in 2019 were from the district of Kerian (Unpublished data, Department of Fisheries). Kuala Gula cages fish farming located in the district of Kerian has been established since 1992. The total of 60 cages operating in Kuala Gula are owned by 60 operators (Unpublished data, DoF). The main species of finfish cultured are seabass, red snapper, grouper, hybrid grouper, mangrove snapper and golden pompano (Annual Fisheries Statistics, 2019).

Microalgae are microscopic organisms that are primary producers in the aquatic systems. However, among 5000 marine microalgae species, 300 species are potentially harmful microalgae (Hallegraeff, 2003). Harmful algal blooms (HABs) take place when microalgae species grow out of control that may cause negative impacts on aquatic life, ecosystems and *human health*. The cell density of microalgae can exceed millions of cells per liter of water that may change the color of waters to reddish, greenish, brownish or pinkish upon the type of microalgae and it is commonly known as “redtide” (Hallegraeff, 2003).

In general, microalgae that possess potential to cause harm could be divided into three groups which are namely toxin producers, high-biomass producer and fish-killing species.. Toxin producer species mainly dinoflagellates such as *Alexandrium* spp., *Dinophysis* spp., *Pyrodinium bahamense var compressum*, *Gymnodinium catenatum* and diatoms, *Pseudo-nitzchia* spp. may cause seafood poisoning to consumers (Hallegraeff, 2003; Omura et al., 2012). The main seafood poisoning associated with different species of microalgae and toxins transmitted by filter-feeders’ organisms like bivalve are Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Amnesic Shellfish Poisoning (ASP), Neurotoxic Shellfish Poisoning (NSP) and Azaspiracid Shellfish Poisoning (AZP). The high-biomass producer such as *Chaetoceros* spp., *Coscinodiscus* spp., *Eucampia* spp., *Rhizosolenia* spp., *Skeletonema* spp., *Thalassiosira* spp. and *Tripos* spp. can create anoxic or hypoxic conditions, water discoloration and cause mortality of marine life (Assmy & Smetacek, 2009; Sakamoto et al., 2021; Sidabutar et al., 2021). Meanwhile, fish killer species that can be harmful to fish and invertebrate by their morphology or their production of mucus or ichthyotoxic compounds such as *Chattonella* spp., *Heterosigma* spp., *Margalefidinium* spp. (Kim & Oda, 2010; Basti et al., 2021) and *Karlodinium* spp. (Place et al., 2012; Lim et al., 2014)

Algal blooms occur naturally and regulated by various environmental changes such as light, temperature, salinity, pH, enrichment of nutrients, anthropogenic activities and climate changes (Nwankwegu et al., 2019; Griffit and Goblera, 2020). Favorable environmental conditions in the water ecosystems will trigger the rapid growth of certain algae species (Anderson et al., 2012; 2015). The sources of nutrients consist of a variety of land-based discharges including agriculture, industrial area, domestis and urban sewage along the lines of inputs sourced from rivers namely aquaculture activities. The continouing presence of nitrogen and phosphate in the water can potentially precipitate eutrophication, a condition of the water becoming nutrient-rich. It has been thought and considered

that nutrient enrichment was the stimulating factor of harmful algal blooms in Jakarta Bay (Sidabutar et al., 2020).

Nationwide, harmful algal blooms have been linked in numerous reports to cause repercussions for the fisheries industry taking in account of the massive death toll on fish mainly towards caged fish as a consequence of the excretion of ichthyotoxic compounds such as Reactive Oxygen Species (ROS) or Poly Unsaturated Fatty Acids (PUFA) which could potentially clog, irritate or damage the fish gills and eventually cause suffocation. (Rensel & Whyte, 2003; Hallegraeff et al., 2018). Blooms of non-toxic algae can lead to fish mortality as mass decomposition of microalgae can deplete dissolved oxygen, creating hypoxic or anoxic conditions (Rensel and Whyte, 2003). In contrast to caged fish, which are trapped in their cages and eventually perish, wild fish are able to swim away from bloom areas that could cause them harm. The species *Chattonella* spp.*, Margalefidinium polykrikoides* and *Karenia* spp. are considered to be, as reported, primarily responsible for the massive blooms of harmful microalgae and accompanying fisheries damages throughout the coasts of East Asia. (Sakamoto et al., 2021). Dinoflagellates *M. polykrikodes* and *Karlodinium* spp. are known as an ichthyotoxic dinoflagellate species that have been recorded to cause massive fish death in many countries including Philippines (Azanza et al., 2008; Yñiguez et al., 2021), China (Lu et al., 2014), Japan (Sakamoto et al., 2021) and Korea (Lee et al., 2013). In Korea blooms of *M. polykrikoides* have become annual events since 1993 (Lee et al., 2013). Diatoms such as *Chaetoceros* spp., *Coscinodiscus* spp., *Eucampia* spp., *Rhizosolenia* spp., *Skeletonema* spp. and *Thalassiosira* spp. have been reported as causative species of red tides in Japan, China, Rusia and Indonesia (Sakamoto et al., 2021; Sidabutar et al., 2021).

In Malaysia, the blooms of *Alexandrium minutum*, *A. tamiyavanichii* (Lim et al., 2012), *Chattonella* sp. (Choo, 1994), *Karlodinium australe* (Lim et al., 2014), *Margalefidinium polykrikoides (*Anton et al., 2008*;* Siti-NorRohaida et al., 2015*)*, *Noctiluca scintillans* (Choo, 1994; Roziawati et al., 2016), *Pyrodinium bahamense var compressum* (Adam et al., 2011), *Tripos furca* (Roziawati et al., 2012) and *Margalefidinium fulvencens* (Roziawati et al., 2022) have been negatively impacted human health, aquaculture and tourism.

Several records of HAB associated with water discoloration and massive fish kill have been documented in the Perak waters since 2007 (Table 1). The first HABs event was recorded in March 2007 in Sungai Dinding, Lumut, Perak coastal waters caused by *T. furca* (DOF, 2007) and again in June 2008 linked with mass death of farmed fish (Roziawati, 2012). The first *M. polykrikoides* blooms in Peninsular Malaysia was reported in Kerian, Perak coastal waters in 2013 associated with mass mortality of cages fish (Siti-NorRohaida et al., 2015). In August 2016, HABs associated mass mortality of caged fish in Kuala Gula, Perak was attributed to the species, *N. scintillans* (Roziawati et al., 2016). The bloom has caused more than RM 500,000 losses.

The baseline data have been limited and scarce regarding potentially harmful microalgae composition and abundance in Kuala Gula, Perak. Research on the composition and abundance of phytoplankton in Perak is beyond doubt should be called for considering the fact of numerous instances of fish deaths in Perak brought on by toxic microalgal blooms. In addition, this region poses a significant role to Malaysian aquaculture productivity. Hence, this study focused on assessing the occurrence and abundance of microalgae in the marine finfish cage culture area of Kuala Gula, Perak. Additionally, the water quality was also investigated in this study. This could be able to provide more information regarding the distribution of harmful algae species within the region as well as contributing to the species inventory for country monitoring purposes.

Table 1 A summary of harmful algal blooms (HABs) events in Perak, Malaysia

Timeline	Harmful algae		Impact	References
March 2007	*Tripos furca*	Sg. Dinding, Perak	Water discoloration	Department of Fisheries, 2007
June 2008	*T. furca*	Sg. Dinding, Perak	Mortality of cultured fish	Roziawati, 2012
March 2013	*Margalefidinium polykrikoides*	Kerian, Perak	Mortality of cultured fish	Siti-NorRohaida et al., 2015; Roziawati and Shahuntala, 2018
August 2016	*Noctiluca scintillans*	Kuala Gula, Kerian, Perak	Mortality of cultured fish	Roziawati et al., 2016
Mei 2020	*M. fulvencens*	Kerian, Perak and Seberang Perai Selatan, Penang	Mortality of cultured fish (RM 11.0 million of losses)	Roziawati et al., 2022
June 2020	*N. scintillans*	Larut Matang & Lumut, Perak	Water discoloration	Unpublished data

Materials & Methods

The study was carried out monthly from Feb 2017 to December 2018 in Kuala Gula fish cages areas located at Kerian district, Perak (Table 2). The climate of the study area can be classified by four main monsoons such as the inter-monsoon 1 (IM1) in April, the southwest monsoon (SWM-dry season) from May to September, the inter-monsoon 2 (IM2) in October and the northeast monsoon (NEM-wet season) from November to March (Suhaila & Jemain, 2012). All sampling stations were established at fish cages. Two sampling stations located further offshore of Sungai Gula (ST. 1, N4°50'11.8", E100°26'25.8" and ST. 2, N4°52'04.5", E100°24'57.1"), one station at Sungai Kelumpang river estuary (ST. 3, N4°55'08.9", E100°29'09.7") and three stations along the Sungai Gula river (ST. 4, N4°55'47.5", E100°28'07.4"; ST. 5, N4°56'23.3", E100°28'13.0" and ST. 6, N4°56'33.7", E100°28'47.2") (Figure 1). A total of 1L of water was sampled at each sampling stations using Van Dorn's sampler in triplicate and then filtered through a net of 20 µm. Samples were then preserved in acidic Lugol's solution.

Temperature, salinity, pH, and dissolved oxygen were among the physicochemical parameters of surface water that were in-situ evaluated in triplicate using a Hydrolab Quanta multiparameter water quality probe (Loveland, CO, USA). Water samples for dissolved inorganic nutrients analyses (nitrate, nitrite, ammonia, phosphate and silicate) were returned to the laboratory and immediately analyzed spectrophotometrically by using a HACH DREL 2010 (HACH, USA). Total suspended solid was analyzed using Photometric Method. Concentration of chlorophyll a (Chl a) was measured *in situ* using a Hydrolab MS5 chlorophyll probe (Loveland, Co, USA).

The microplankton identification was carried out according to Tomas (1997), Backer et al., (2003) and Omura et al., (2012). The micrograph for each microplankton was taken using a CCD camera and Analysis (R) software (Soft Imaging System Inc., USA). A concentrated microplankton sub-sample (1ml) were placed in the counting chamber of Sedgewick-Rafter and enumerated under Compound Microscope (Leica, USA) at 100 times magnification. Three replications were used to calculate the cell density, or cell L-1, for each microplankton.

Using GraphPad Prism version 6.0, data on chl a, microplankton, and physico-chemical readings were processed, and graphs were produced. To compare the various phytoplankton groups

on a broad scale, data were presented as microplankton composition (%). The importance of each variable (temperature, pH, salinity, dissolved oxygen, total suspended solids, turbidity, nitrite, nitrate, ammonia, phosphate, chlorophyll a, and cell density) was assessed using Prism's Analysis of Variance (ANOVA) function. The correlation between the environmental variables and the cell densities of microphytoplankton was examined using principle component analysis (PCA). To test the importance of the axes, PCA was done using the PC-ORD programme (McCune and Mefford, 2006) with 1000 permutations.

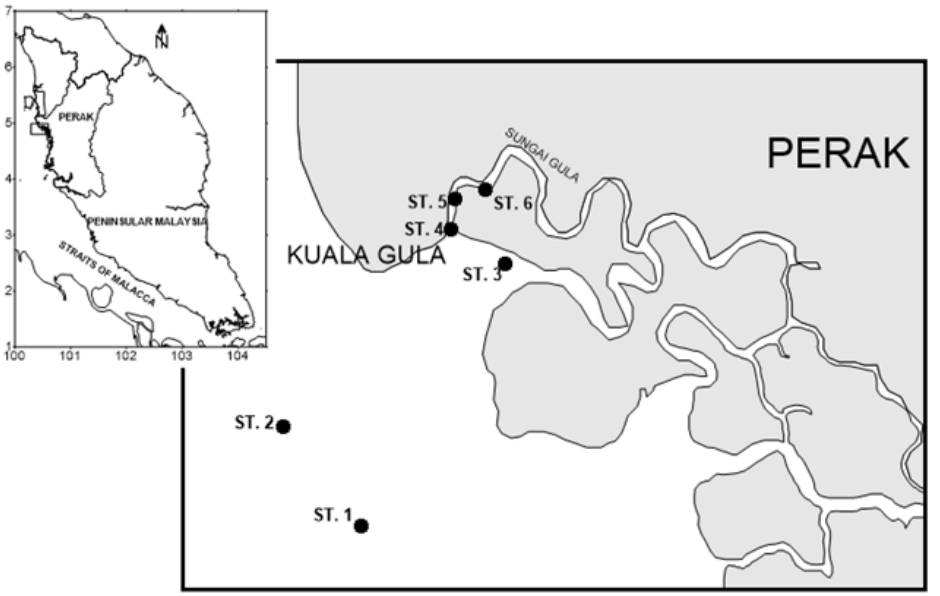

Figure 1. Maps of Kuala Gula, Perak showing the six sampling stations of fish cages areas from February 2017 to December 2018

Table 2. Sampling dates during the study duration in Kuala Gula, Perak

Sampling Number	Date	Season
1.	21 February 2017	Northeast Monsoon (NEM)
2.	28 March 2017	Northeast Monsoon
3.	20 April 2017	Intersessional Monsoon (IM1)
4.	22 May 2017	Southwest Monsoon (SEM)
5.	20 June 2017	Southwest Monsoon
6.	20 July 2017	Southwest Monsoon
7.	17 August 2017	Southwest Monsoon
8.	18 September 2017	Southwest Monsoon
9.	16 October 2017	Intersessional Monsoon (IM2)
10.	15 November 2017	Northeast Monsoon
11.	22 December 2017	Northeast Monsoon
12.	15 January 2018	Northeast Monsoon
13.	26 February 2018	Northeast Monsoon

14	28 March 2018	Northeast Monsoon
.	14 May 2018	Southwest Monsoon
15	26 June 2018	Southwest Monsoon
.	20 August 2018	Southwest Monsoon
16	29 October 2018	Intersessional Monsoon
.	21 November	Northeast Monsoon
17	2018 20	Northeast Monsoon
.	December 2018	
18		

Results & Discussions

Phytoplankton composition and abundance

A total of 64 taxa of microalgae recorded consist of three main groups, i.e. diatoms (45 genera), dinoflagellates (17 genera) and cyanobacteria (2 genera). The abundance of microplankton and chlorophyll a concentration in all sampling stations showed fluctuation throughout the sampling period (Figure 2). Analysis of Variance (ANOVA) shows significant difference ($P<0.0001$) among months for cell density of microplankton and chlorophyll a concentration. The cell density of microplankton in the study were ranged from 2.73 × 10cells L-1 to 1.5 × 10 cells L-1. The highest cell density of microplankton was observed in October 2018 at St. 1, while the lowest in January 2018 at ST. 5. Concentration of chlorophyll a ranged from 0.85 µg L-1 (December 2018, ST. 3) to 41.13 µg L-1 (June 2016, ST. 4). Abundance of microplankton more than 107cells L-1 is categorized as algal blooms (Pasztaleniec & Poniewozik, 2010; Veronica et al., 2014). However, bloom of microplankton was not observed at all sampling stations during the period of study throughout the sampling period.

Figure 3 and 4 shows dinoflagellates and diatoms documented at Kuala Gula, Perak. Diatoms were found dominant at all sampling stations of more than 71.0 % of the total microalgae throughout the sampling period except in February 2017 at ST. 6 (30.3 %), November 2018 at ST. 3 (55.3 %) and in December 2018 at ST. 6 (58.6 %) (Figure 5). Whereas, the percentage of dinoflagellates is low throughout the study period covering 0.06 % to 28.4 % of total phytoplankton except in February 2017 at ST. 6 (69.7 %), November 2018 at ST. 3 (44.6 %) and in December 2018 at ST. 6 (41.2 %). Different species of diatoms were dominant in each sampling occasion at all sampling stations. *Thallasiosira* spp. was dominant at ST. 1 and ST. 2 throughout March 2017 (80.7 % and 85.4 %) until May 2017 (56.6 % and 69.0 %), ST. 3 and ST.4 from July 2017 (73.2 % and 92.2 %) to November 2017 (77.64 % and 74.4 %) and ST. 5 and St. 6 in August 17 (98.0 % and 96.0 %), October 17 (77.2 % and 60.0 %). *Skeletonema* spp. was found dominant several times at all sampling station except at St. 1 and ST. 2. *Skeletonema* was found dominant once at ST. 3 in August 2018 (88.0 %), twice at ST. 4 (February 2017 and November 2018) and ST. 5 (November 2018 and December 2018) and several times at ST.6 (April-May 2017, February- -May 2018 and November 2018). *Bacillaria* spp. was only observed dominant twice in June 2017 at ST. 3 and St. 4 (69.9 % and 57.5%) and August 2018

cells L-1, 2.0 × 102 cells L-1 and 2.4 × 105 cells L-1, respectively (Table 3). Some species of *Alexandrium* such as *A. minutum* and *A. tamiyavanichii* are known as saxitoxin producers and have been associated with paralytic shellfish poisoning (PSP) cases in Malaysia (Mohammad-Noor et al., 2017; Usup et al., 2002). In some countries, the presence of the saxitoxin producing species in the concentration of up to 1.0 × 103 cells L-1 requires further toxin analysis in shellfish (Anderson et al., 2001). Diarrheic shellfish poisoning (DSP) toxins are produced by the dinoflagellates *Dinophysis* and *Prorocentrum* (Lee et al., 2016). Several species of *Dinophysis* such as *D. acuminata, D. acuta, D.caudata, D. fortii, D. miles, D. ovum, D. sacculus* are reported to produce diarrheic shellfish toxins (DST) and pectenotoxins (PTXs) and have been associated with DSP events in Europe, Chile, Japan, and New Zealand (Reguera et al., 2012) and *Prorocentrum donghaiense* and *P. lima* in China (Chen et al., 2013; Yin et al., 2018). Okadaic acid has been detected in phytoplankton samples (500 cells of *Dinophysis caudata*) and green mussels from the Singapore's part of the Straits of Johor (Holmes et al., 1999). There have been no reports of DSP in Malaysia waters to date. In Malaysia, a toxic bloom of *Pseudo- nitzschia cuspidata* (5.6 × 105 cells L-1 to 3.5 × 106 cells L-1) was first reported in Miri, Sarawak, and the low level of neurotoxin domoic acid was detected in shellfish and phytoplankton samples (Teng et al., 2021). Blooms of *Pseudo-nitzschia* (7.29 × 105 cells L-1 to 1.14 × 106 cells L-1) has also been attributed to oxygen depletion in waters and mass fish kill event in Puducherry, India (Mishra et al., 2021).

In this study, the potentially blooming species and fish killer species found were *Akashiwo sanguinea*, *Chaetoceros* spp., *Noctiluca scintillans, Prorocentrum micans, Tripos furca*, *T. fusus* and *Skeletonema* spp. *Akashiwo sangunea*, *Cheatoceros* spp., *Prorocentrum micans* and *T. furca* are commonly found in each sampling work at all sampling stations. Species of *Akashiwo sanguinea, T. furca* and *T. fusus* were commonly found in coastal water off Malacca (Mohammad-Noor et al., 2007), Sg. Jarum Mas, Perak (Roziawati & Faazaz, 2011), Tebrau Strait (Lim et al., 2014) and Pulau Aman, Penang (Roziawati et al., 2015).

Dinoflagellates, *Akashiwo sanguinea* has caused blooms in coastal waters worldwide such as Australia, North and South America, Europe and Asia associated with mass mortality of fish, shellfish and birds (Wu et al., 2022). *Akashiwo sanguinea* has caused a large fish kill along Bolivar Peninsula, Texas in 2007 (Antonietta, 2016). *Tripos fusus* has caused water discoloration and mass mortality of marine organisms in the coastal of water Oman (Al Gheilani et al., 2011). Red discoloration of Sungai Dinding, Lumut, Perak coastal waters has been reported in 2007 caused by blooms of *T. furca* with cell density ranged from 2.0 × 106 cells L-1 to 1.5 × 108 cells L-1 (DOF, 2007) and again in June 2008 with maximum cell density of 4.6 × 105 cells L-1 (Roziawati et al., 2012). *Chaetoceros* spp. have been reported to have caused fish mortality in fish cages (Rensel and Whyte, 2003; Helleren, 2016). The cell densities of *Chaetoceros* spp. at 7.48 × 105 cells L-1 has been associated to fish mortality incident in Cockburn Sound, Western Australia, (Helleren, 2016). In the present study, *Chaetoceros* spp. was recorded in highest density at 3.49 × 104 cells L-1 in June 2017 at St. 2. *Prorocentrum micans* bloom has been reported to cause fish or shellfish mortality due to oxygen depletion or anoxia (Cho et al., 2009; Yin et al., 2018).

Dinoflagellate, *Karlodinium* spp. and *Margalefidinium* spp. were rarely found throughout the sampling period, relatively in low density. Fish kills events in Tanjung Kupang, Johor fish cage cultures was reported in February 2014 attributed to the *Karlodinium australe* with cell densities up to 1.25 × 106 cells L-1 (Lim et al., 2014) and recurred in February 2015 at cell densities up to 2 × 108 cells L-1 with losses of over 30 tons of cultured fish (Teng at al., 2016). *M. polykrikoides* have been reported to cause fish kill in many countries such as Mexico (López-Cortés, 2019), the Philippines (Azanza et al., 2008), Korea (Baek et al., 2020), Japan (Sakamoto et al., 2021), United States (Anderson et al.,

2021). In Malaysia, *M. polykrikoides* was first reported in Sepanggar Bay, off Kota Kinabalu, Sabah in 2005 and since then its blooms have become regular and annually observed in the waters (Anton et al., 2008; Adam et al., 2011). During the event, the density of *M. polykrikoides* reached up to 6.0 × 106 cells L-1 triggering fish mortality. In March 2013 the bloom of *M. polykroikodes* occurred in Kerian and Kuala Sepetang, Perak fish farms (Siti-NorRohaida et al., 2015; Roziawati & Shahuntalla, 2018). No fish kill event due to microalgae blooms was recorded throughout the sampling period of the present study. However, the latest event of HAB associated with massive fish deaths has been reported in the coastal waters of Kerian, Perak and Penang in May 2020 due to *Margalefidinium fulvencens* caused huge losses to farmers (Roziawati et al., 2022).

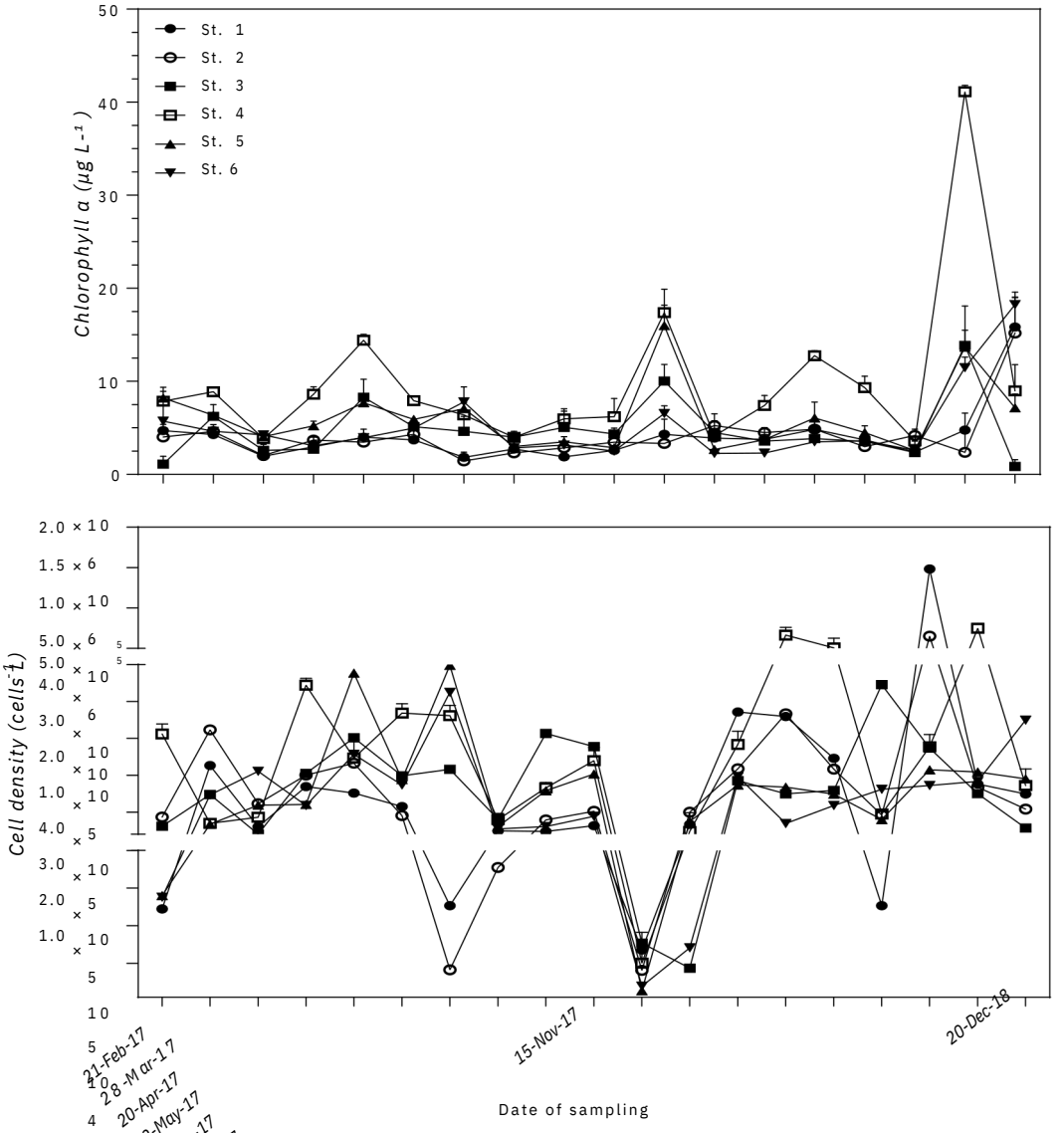

Figure 2. Chlorophyll a and cell density of phytoplankton in six sampling stations of Kuala Gula, Perak from February 2017 to December 2018.

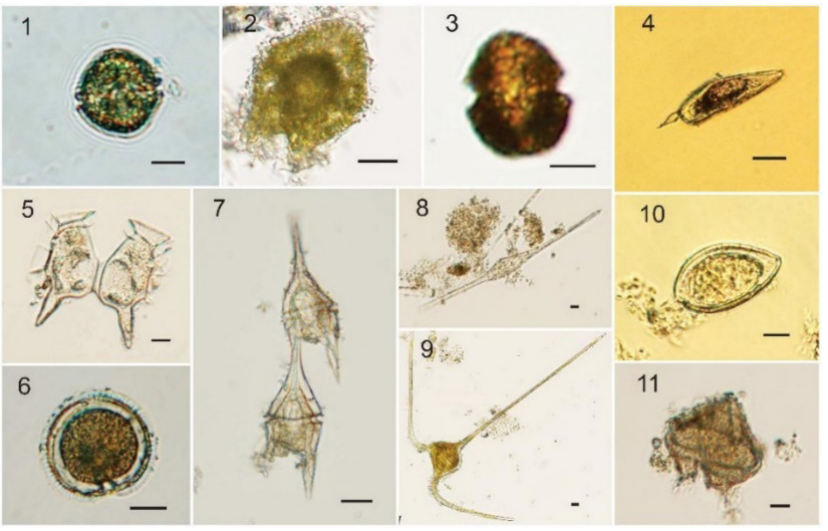

Figure 3. Micrographs of dinoflagellates found in Kuala Gula, Perak. 1. sp. 2. *AlexandriumAkashiwo sanguinea*, 3. *Karlodinium* sp. 4. *Prorocentrum gracile*, 5. *Dinophysis caudata*, 6. *Pyrophacus* sp., 7. *T. furca*, 8. *T. trichocerus*, 10. *P. micans*, 11. *Protoperidinium* sp. Scale bar: All 20 µm except 3 and 10 are 10 µm.

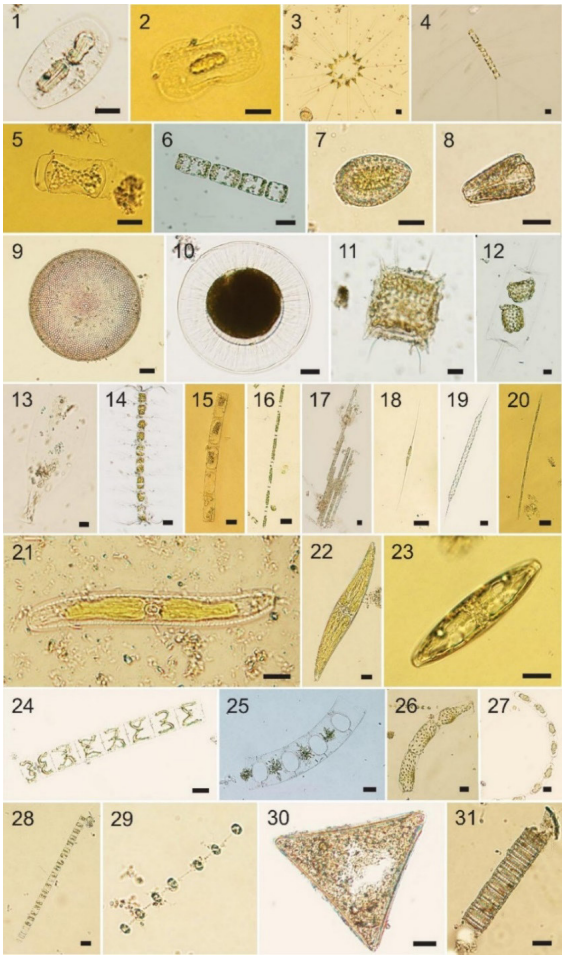

Figure 4. Micrographs of diatoms found in Kuala Gula, Perak. 1. *Amphora* sp., 2. *Amphiprora* sp., 3. *Asterionella* sp., 4. *Chaetoceros* sp., 5. *Melosira* sp., 6. *Lauderia* sp., 7. *Surirella* sp., 8. *Licmophora* sp., 9. *Coscinodiscus* sp., 10. *Planktionella* sp., 11. *Odontella* sp., 12. *Ditylum* sp., 13. *Helicotheca* sp., 14. *Bacteriastrum* sp., 15. *Bellerochea* sp., 16. *Leptocylindricus* sp., 17. *Bacillaria* sp., 18. *Cylindrotheca* sp., 19. *Rhizosolenia* sp., 20. *Pseudo- nitzschia* sp., 21. *Gyrosigma* sp., 22. *Pleurosigma* sp., 23. *Navicula* sp., 24. *Melosira* sp., 25. *Eucampia* sp., 26. *Guinardia* sp., 27. *Hemiaulus* sp., 28. *Skeletonema* sp., 29. *Thalasssiosira* sp., 30. *Triceratium* sp., 31. *Paralia* sp., Scale bar: All 20 µm except 6 is 10 µm

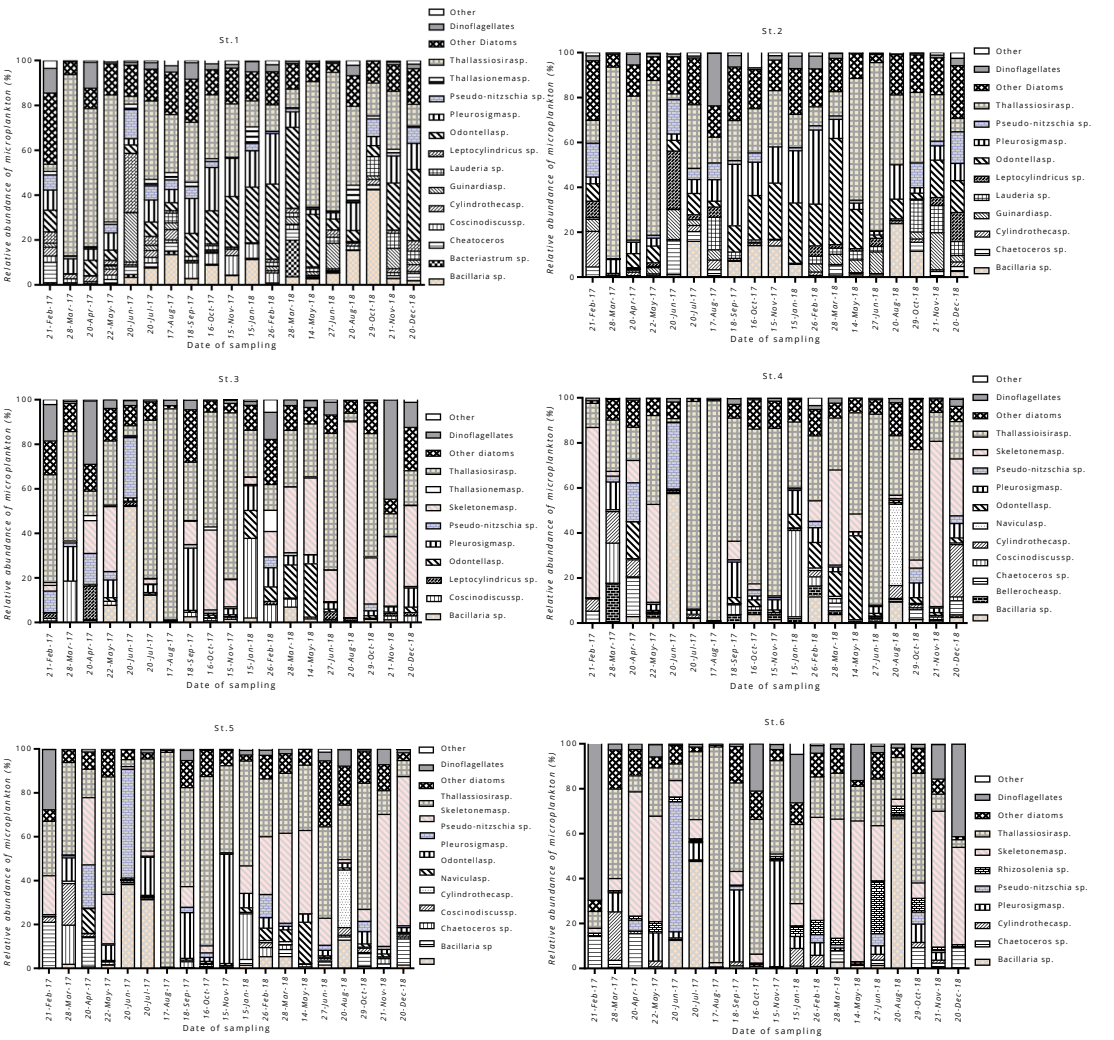

Figure 5. Relative abundance of microplankton in six sampling stations of Kuala Gula, Perak from February 2017 to December 2018.

Table 3. List of potentially harmful microalgae found in Kuala Gula, Perak from February 2017 to December 2018.

Species	Maximum density (cells L-1)	Frequency	Potential Impact (Reference)
Potentially toxic species			
Alexandrium spp.	7.6 ×	1	PSP (Horner et al., 1997)
Dinophysis caudata	103 1.0	8	DSP (Reguera et al, 2012)
D. acuminata	× 103	1	DSP (Reguera et al, 2012)
Pseudo-nitzchia spp.	2.0 ×	8	ASP (Lee et al., 2016)
Bloom forming species	102 2.4	1	
	× 105	1	
		9	

Akashiwo sanguinea	1.3	×	1	Red tides (Mania and Rose, 2002)
Chaetoceros spp.	103	3.5	2	Red tides, fish kill (Horner et al., 1997)
Margalefidinium spp.	×	104	1	Red tides, fish kill (Kim & Oda, 2010)
Noctiluca scintillans	1.3	×	8	Fish kill (Mania and Rose, 2002)
Skeletonema spp.	102	4.3	2	Red tides, fish kill (Sidarbutar, 2021)
Prorocentrum micans	×	103	1	Red tides (Horner et al., 1997)
Tripos furca	5.5	×	2	Red tides, fish kill (Horner et al., 1997)
T. fusus	105	9.7	1	Red tides, fish kill (Horner et al., 1997)
	×	102	9	
	4.9	×	1	
	103		8	
	5.7×		1	
	102		7	
			1	
			8	

Physical and chemical properties of seawater

Figure 6 shows monthly rainfall trend of Bagan Serai, Perak that were obtained from Malaysian Metrological Services. The least rainfall amount was recorded in July 2017 (22.5 mm) and June 2018 (77.0 mm), both in the southwest monsoon season. Meanwhile the higher amount was recorded in September 2017 (508.7 mm) and October 2018 (494.2 mm) in inter-monsoon. Based on the number of monthly rainy days, the least occurrence was recorded in July 2017 (5 days) and July 2018 (4 days) while in October (22 days) and December 2018 (21 days) the rainy days were the higher. October belongs to inter-monsoon, the beginning of the Northeast Monsoon which was received more rainfall similarly reported by Hanif et al. (2022) that stated higher rainfall occurred in October.

The values of physico-chemical parameters at different sampling stations in Kuala Gula, Perak are presented in Figure 7. All physico-chemical parameters show distinct monthly variation among different sample sites and different sampling time. Seawater temperature was ranged from 27.50°C (January 2018 at ST. 5) and 31.67°C (February 2017 at ST. 6). Temperature declined from May 2017 to January 2018 and slight increase in February 2018. The significant amount of rain that fell during the Northeast Monsoon may be responsible for the low temperature. Total suspended solid (TSS) were below the Marine Water Quality Standard for Malaysia (MWQS) of 150 mg L-1 at ST. 1 and ST. 2 throughout the sampling period. However, TSS reading exceeded the MWQS in March 2017 at ST. 3, ST. 4 and ST. 5, May 2017 at ST. 5, March 2018 at ST. 3 and May 2018 at ST. 4. The highest TSS was recorded in March 2017 at ST. 4 (565 mg L-1) and the lowest in February 2018 at ST. 2 (5.00 mg L-1).

One-way ANOVA shows significant difference ($P<0.05$) among stations for pH, salinity and dissolved oxygen (DO). The values of pH recorded at all locations were within the recommended pH (6.5 to 9.0) for optimal fish production with ranged from 6.75 (ST. 6 January 2018) to 8.68 (ST. 2, July 2017). Salinity along the Sungai Gula river (ST. 4, ST. 5 & ST. 6) was in the range of 15.38 ppt to 30.28 ppt, Sungai Kelumpang (ST. 3) was ranged between 22.46 ppt to 29. 87 ppt and two other sampling locations further offshore of Sungai Gula (ST. 1 & ST. 2) was ranged between 26.61 to 31.72 ppt. In most sampling points, salinity decreased during months with more precipitation (i.e. September 2017, January 2018 and October 2018). Dissolved oxygen (DO) of water at ST. 1 and ST. 2 were mostly greater than 5.00 mg L-1 with a mean of 5.49 mg L-1 (range 3.74-7.25 mg L-1). DO in ST. 3 had a mean of 4.57 mg L-1 (range 2.00-5.61 mg L-1). Meanwhile, DO along the river of Kuala Gula mostly was less than 5.00 mg L-1 with a mean of 3.65 mg L-1 (range 1.15-6.32 mg L-1).

One-way ANOVA shows significant difference ($P<0.05$) among stations for nitrite, nitrate, ammonia, phosphate and silicate. Nitrate and nitrite ranged from 0.00 mg L-1 to 1.23 mg L-1 and 0.002 mg L-1 to 0.93 mg L-1, respectively. Nitrate level in ST. 1 and ST. 2 were mostly below the MWQS of 0.06 mg L-1. However, nitrate level in May 2017, January 2018, August 2018 and October 2018 at all

sampling stations have exceeded the MWQS. High concentration of nitrate was recorded in the most occasions at the river of Kuala Gula. Ammonia level in all sampling stations except ST. 1 and ST. 2 mostly exceeded the MWQS of 0.07 mg L-1 with ranged from 0.00 mg L-1 to 0.36 mg L-1. Nitrogen in the forms of nitrate, nitrite, or ammonium enters waterway from several sources including industrial wastes, sewage effluents, run off from agriculture and aquaculture activities (Anderson et al, 2002; Davies and Ugwumba, 2013). The effluence from fish farm waste may be the reason why ammonium concentrations were greater at sample points close to the fish cages. Along the Sungai Gula and Sungai Kelumpang rivers are also shrimp farms protected by mangrove forests. Unconsumed fish meals and waste from fish excrement are released into the environment by aquaculture activities, particularly fish cages (Yin et al., 2008).

Phosphate concentrations were mostly higher than the acceptable values of MWQS of 0.075 mg L-1 at all sampling stations throughout sampling time with ranged between 0.00 mg L-1 and 0.67 mg L-1. Lamoljo et al., (2009) found the concentrations of soluble reactive phosphorus (SRP), nitrate nitrogen (NO2 -N), total ammonia nitrogen (TAN) at Kuala Gula waters in year 2007 (June to September) were below the recognized water quality standards with maximum value of 0.056 mg L-1, 0.086 mg L-1, 0.086 mg L-1, respectively. Concentration of silicate was ranged from 0.01 mg L-1 to 7.25 mg L-1.

It was discovered that the microalgae count was greater in October 2018 and decreased in January 2018. The majority of sample stations reported a rise in microalgae in the months with more precipitation (i.e., May 2018, October 2018, and December 2018), and this pattern followed that of the ammonia, nitrate, and phosphate contents, which all increased. The proliferation of phytoplankton may have been spurred by an increase in nitrogen input into water systems from inland runoffs during periods of heavy rainfall. HABs episodes typically happen after a period of intense rain followed by a period of intense sunlight caused an increase in nutrients in the water column, which acts as a trigger for bloom (Smadya, 2003).

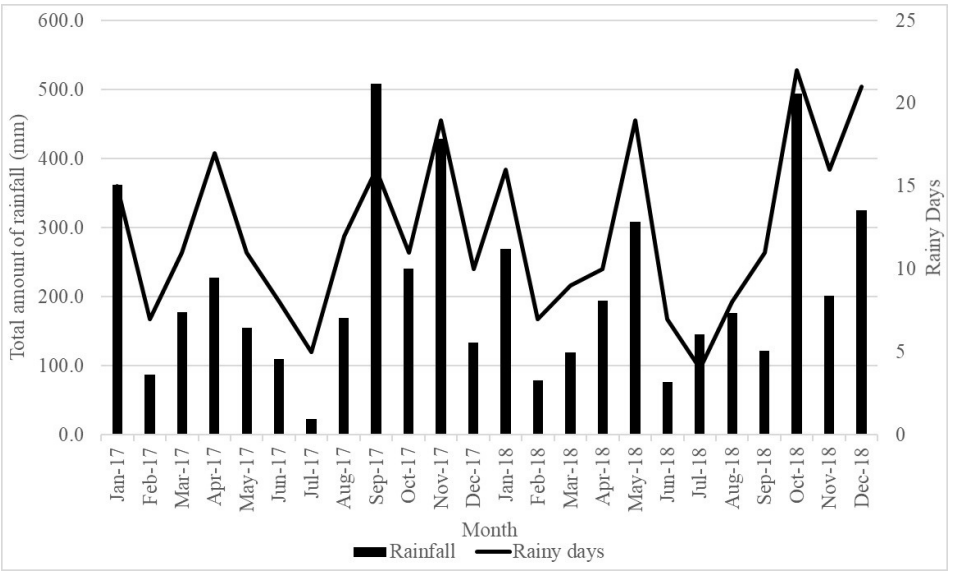

Figure 6: Total monthly rainfall and rainy days in Bagan Serai, Perak from January 2017 to December 2018 (Source: Department of Meteorological Malaysia).

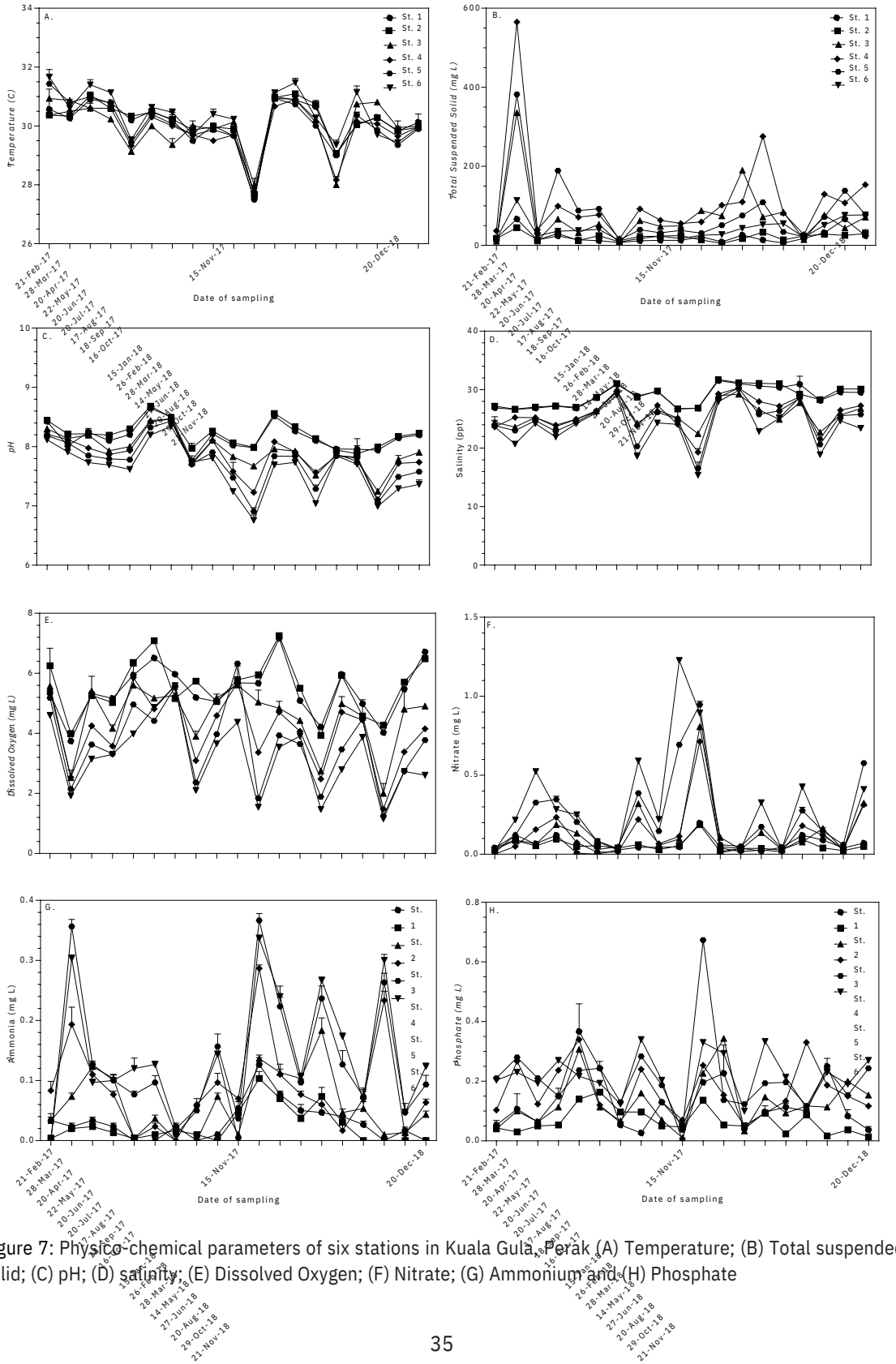

Figure 7: Physico-chemical parameters of six stations in Kuala Gula, Perak (A) Temperature; (B) Total suspended solid; (C) pH; (D) salinity; (E) Dissolved Oxygen; (F) Nitrate; (G) Ammonium and (H) Phosphate

Relationship between cell abundance of microphytoplankton and the environmental variables

Principle component analysis (PCA) was used to analyse the connection between the environmental variables and the cell densities of microphytoplankton. The dataset from Kuala Gula's six sampling stations was analysed over the course of 20 months. Salinity, seawater temperature, dissolved oxygen (DO), pH, amounts of phosphate, nitrate, nitrite, silicate, and ammonia are among the environmental factors analysed. Result of PCA showed that relationships among eleven variables were best explained by the first two axes (Axis 1 & 2) with the total variance of 46.92 %. The biplot result (Figure 8) showed that cell density of microphytoplankton in Kula Gula was positively correlated with phosphate, silicate and ammonia. However, the cell density of microphytoplankton has negative correlation with nitrate, nitrite, temperature, pH and salinity. Increased phytoplankton abundance is a result of nutrient enrichment, which has a considerable impact on phytoplankton growth. (Paul et al., 2008). However, no blooms of microalgae were recorded throughout the study period in all sampling stations.

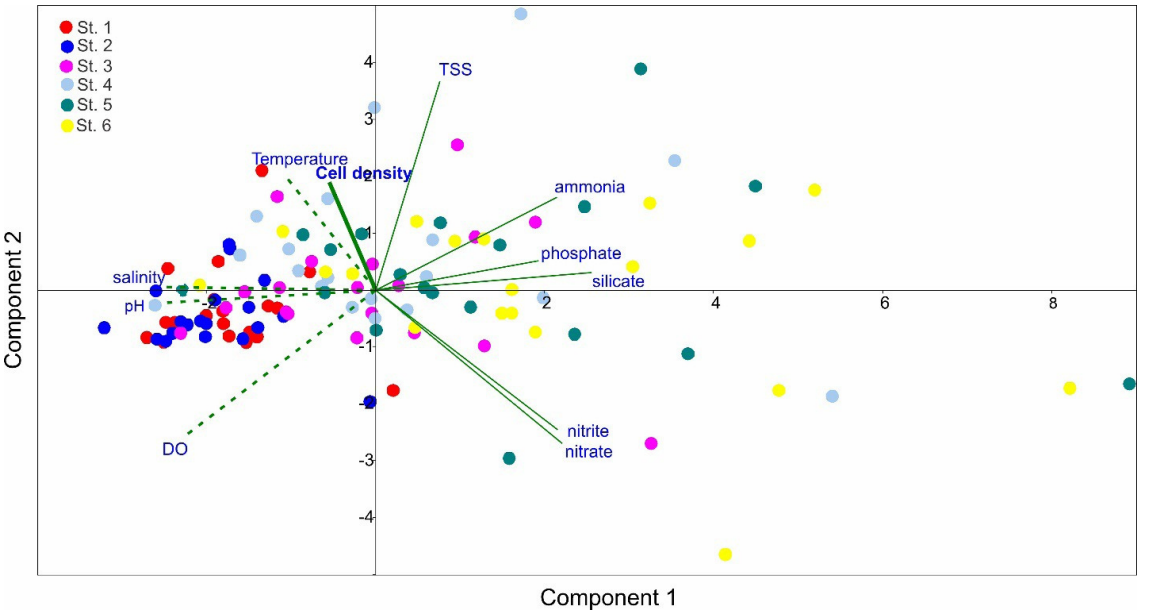

Figure 8. Principal Component Analysis (PCA) generated from ten environmental variables and cell densities of microphytoplankton from Kuala Gula, Perak

Conclusions

Throughout the sample period, diatoms were discovered to be prevalent at all sampling stations. During the study period, no sample station recorded any microalgae blooms. The presence of potentially toxic species such as *Alexandrium* spp., *Dinophysis caudata*, *D. acuminata* and *Pseudo-nitzchia* spp. as well as fish killing species such as *Akashiwo sanguinea*, *Karlodinium* sp., *N. scintillans, Margalefidinium* sp., *Tripos furca* and *T. fusus* were recorded at all sampling stations. The impact of HAB incidence on aquaculture has been evidenced in 2013, 2016, and 2020 in Kuala Gula that caused massive fish death. The sudden presence of HAB species without monitoring and early detection could potentially threaten the aquaculture industry. Thus, continuous microalgae monitoring through remote monitoring should be implemented to provide early warning of HAB in order to safeguard mariculture industries in Perak especially in Kuala Gula.

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Distribution and Density of Edible Bivalve and Gastropods Naturally Occurring in the Coastal Waters of Perak, Malaysia

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Abstract: This study was conducted to identify the distribution and density of bivalve of edible bivalve and gastropods such as the Asiatic hard clam, inequivalve ark, olive whelk, tiger moon snail, murex shell and the spiral melongena found naturally in the coastal waters of Perak, Malaysia. The sampling locations covered the entire Perak coastline from Tanjung Piandang to Hutan Melintang. Sampling was conducted using a long dragged scoop and the distance from the shoreline was limited to less than five nautical miles. The results from this study showed that main area for the Asiatic hard clam was at the sandbar area of Tanjung Beras Basah near the river mouth of Sungai Perak. The density of Asiatic hard clam there ranged between 458 to 3,551 kg.km-2. As for the inequivalve ark, this species can be found at three main areas namely Kuala Kurau-Kuala Gula, Pulau Pasir Hitam and Pantai Remis-Segari area. The densities of inequivalve ark populations ranged from 49 to 20,762 kg.km-2. While the distribution of olive whelk was mainly found in the northern region of Tanjung Kupang, at the river mouth of Sungai Perak (density of 2,037 kg.km-2) and at the river mouth of Sungai Bernam at Tanjung Beras Basah (density of 1,113 kg.km-2). The distribution of tiger moon snail, murex shell and spiral melongena are related to the mangrove habitat and can be found mainly at the northern Perak region from Tanjung Piandang to Pantai Remis. The average density for the three species were 313±129, 228±28 and 629±125 kg.km-2 (±SE) respectively.

Keywords: bivalve, gastropods, Perak waters, density, distribution, capture fishery

Abstrak: Kajian ini dijalankan bagi mengenal pasti taburan dan kepadatan kerang-kerangan dan gastropod yang boleh dimakan seperti kepah, kerang bulu, siput Nassa, siput bintang, siput duri dan siput unam di perairan pantai di Perak, Malaysia. Lokasi persampelan merangkumi seluruh perairan pantai Perak daripada Tanjung Piandang sehingga Hutan Melintang. Persampelan dijalankan menggunakan alat tangguk bergalah dan jarak daripada pantai dihadkan kepada kurang daripada lima batu nautika. Keputusan kajian ini menunjukkan taburan bagi kepah tertumpu di kawasan beting pasir di Tanjung Beras Basah berhampiran muara Sungai Perak. Kepadatan kepah di sana berjulat antara 458 hingga 3,551 kg.km-2. Bagi kerang bulu pula, spesies ini boleh dijumpai di tiga kawasan utama iaitu di Kurau-Kuala Gula, Pulau Pasir Hitam dan Pantai Remis-Segari. Kepadatan populasi kerang bulu berjulat di antara 49 to 20,762 kg.km-2. Manakala taburan siput Nassa boleh dijumpai di kawasan utara Tanjung Kupang, di muara Sungai Perak (kepadatan 2,037 kg.km-2) and dan di muara Sungai Bernam di Tanjung Beras Basah (kepadatan 1,113 kg.km-2). Taburan bagi siput bintang, siput duri dan siput unam pula adalah berkait dengan habitat bakau dan boleh dijumpai di kawasan utara Perak daripada Tanjung Piandang sehingga ke Pantai Remis. Purata kepadatan bagi ketiga-tiga spesies ini masing-masing ialah 313±129, 228±28 dan 629±125 kg.km-2 (±SE).

Introduction

The marine bivalve resources are essential supply of food sources in Malaysia. Malaysians in general consume some of the highest amount of fish protein in the world, with average per capita fish consumption of more than 50 kg per year (FAO, 2018). The main sources of fish protein in Malaysia come from local capture fisheries and aquaculture. The waters of Perak state in Peninsular Malaysia is a very productive area for marine bivalve and gastropods as there are several river mouths (such as Sungai Kurau, Sungai Perak and Sungai Bernam) that supply freshwater and nutrients into the surrounding estuaries. Local fishermen as well as aquaculturists ply their trade in these productive waters and marine mollusks can be derived from those activities.

Perak is also home to over 40,288 hectares of mangrove swamp area (Zulfa et al., 2021). The mangrove habitat is part of the blue ecosystems (along with saltmarshes and sea grasses), is one of the largest carbon pool and able to capture four times more than the rainforests (Rozainah and Sahadev, 2020). The mangrove forest sequestered large amount of carbon in the form of mangrove litter, which in turn contributes to the detritus food chain and support the available bivalve populations (Chong, 2007; Niiyama et al., 2012). Distribution of mangrove forest in Perak mainly focused in the northern region from Kuala Gula to Pantai Remis. Minor patches in the south near the river mouths of Sungai Bernam and Sungai Perak (Hamdan and Muhamad Afizzul, 2020).

The muddy substrate in the coastal mangrove area of Perak is also very conducive to the growth of certain bivalve organisms such as blood cockle (Nakao et al., 1989; Kamaruzzaman et al., 2008; Wan Rasidah et al., 2015). Among the mollusks of commercial importance which can be found in these waters are the blood cockle (*Tegillarca granosa*) or locally known as 'kerang', the Asiatic hard clam (*Meretrix meretrix*) or locally known as 'kepah', murex shell (*Murex* spp.) or locally known as 'siput duri' and the spiral melongena (*Pugilina* sp.) or locally known as 'siput unam'. Other edible mollusks available in Perak are inequivalve ark (*Anadara inaequivalvis*) or locally known as 'kerang bulu', olive whelk (*Nassarius olivaceus*), tiger moon snail (*Natica tigrina*) or locally known as 'siput bintang'.

In relation to that, Perak state is also recognized as the primary producer of blood cockle in Malaysia with annual output of 14,834 metric ton or 79% of the total production in the country (DOFM, 2020). Together with Selangor, these two states produced the most blood cockle in Malaysia. However, blood cockle production has been on the decline in Perak. In the span of two decades, blood cockle production in Perak has decreased from 56,032 metric ton in year 1999 to 26,387 metric ton in year 2010, and currently at 14,834 metric ton (DOFM, 1999; DOFM, 2010; DOFM, 2020). Most of the blood cockle production from Perak are from coastal aquaculture lots and the blood cockle seeds sown in the culture lots could be sourced locally or from other states (Choo and Raihan, 2000). In contrast, other species such as the Asiatic hard clam, murex shell, inequivalve ark, tiger moon snail, olive whelk and the spiral melongena are naturally occurring populations which may have fluctuations in population sizes due to environmental and biological causes.

There were many studies conducted pertaining to the subject of bivalve in Perak waters, especially concerning the cultured blood cockle species in the mangrove area. Such studies focused on topics such as the blood cockle biology and the associated environmental conditions (Yurimoto et al., 2014), mangrove food chain relationship (Niiyama et al., 2012) and the impact of water quality (Shahunthala, 2015; Amirul Azuan et al., 2021). Other studies on bivalve and gastropods in general also focused on the water quality aspects such as the sanitation and food safety of cultured shellfish

(Wan Norhana et al., 2011) and the effect of antifouling paint on cockle (Mukhtar et al., 2020). A recent study by Lai et al., (2020) was conducted to assess the macrobenthic community associated with the blood cockle culture area but only focused on one area in northern Perak.

However, little information is available on the resource status of other bivalve and gastropod species in the same area, which could otherwise assist in effective fisheries management for the sustainable utilization of available bivalve and gastropods resources. Therefore, this study aimed to improve on the current available information of bivalve and gastropods resources in Perak waters and focused specifically on the distribution and the density of the bivalve and gastropods communities occurring naturally in the area was conducted. The objectives of this study were to identify the distribution of commercially important bivalve and gastropods naturally available at the coastal area of Perak state and to determine the densities of selected species such as the Asiatic hard clam, inequivalve ark, olive whelk, tiger moon snail, murex shell and the spiral melongena.

Materials and Methods

Two sampling sessions were carried out in year 2019 and 2020. The first sampling was conducted on 20 August to 22 November 2019 (northern and southern Perak) while the second sampling was conducted on 25 August to 18 September 2020 (central region of Perak). The sampling locations were selected parallel to the shoreline (less than 5 nautical miles) and positioned randomly from one another so as to cover the entire Perak coastline during the duration of this study.

During the samplings, bottom organisms were collected using a long-dragged scoop (which is normally used by blood cockle harvesters) onboard a specially rented fisherman's boat. The coordinates of the sampling stations were recorded using a handheld GPS device (GARMIN Montana 650) before the commence of each sampling operation. For each sampling session, two types of long dragged scoops with different mesh sizes were used (coarse mesh opening of 20mm and fine mesh opening of 5mm) (Figure 1). However, for the sampling session in the Tanjung Piandang-Kuala Terong waters, only the coarse meshed long dragged scoop was used.

The duration for each haul was set at approximately 60 seconds or according to the limit of the dredge basket capacity. The distance travelled during each sampling operation was recorded from the GPS device. Collected samples were stored in plastic bags, tagged and brought back to the jetty for composition and density analyses. On land, the samples were then rinsed, sorted according to major groups, identified to the genus/species level and had their length and weight measured. The density of each species was calculated by dividing the biomass of collected specimens by the area covered by the long-dragged scoop during each sampling operation:

Density (kg.km-2) = Biomass (kg) / Area covered by dredge (km2)

Where:
Area (km2) = length of long dragged scoop opening (m) x distance travelled (km) / 1000

The density results for each selected species in this study were mapped out graphically using the Surfer™ 8 software. For this study, a total 55 stations were sampled in year 2019 while another 52 stations were sampled in year 2020 (Figure 2).

Figure 1. Two types of long dragged scoops with different mesh size were used in this study (left: coarse mesh size of 20 mm, right: fine mesh size of 5mm). The length of the long-dragged scoop opening was measured for density calculation

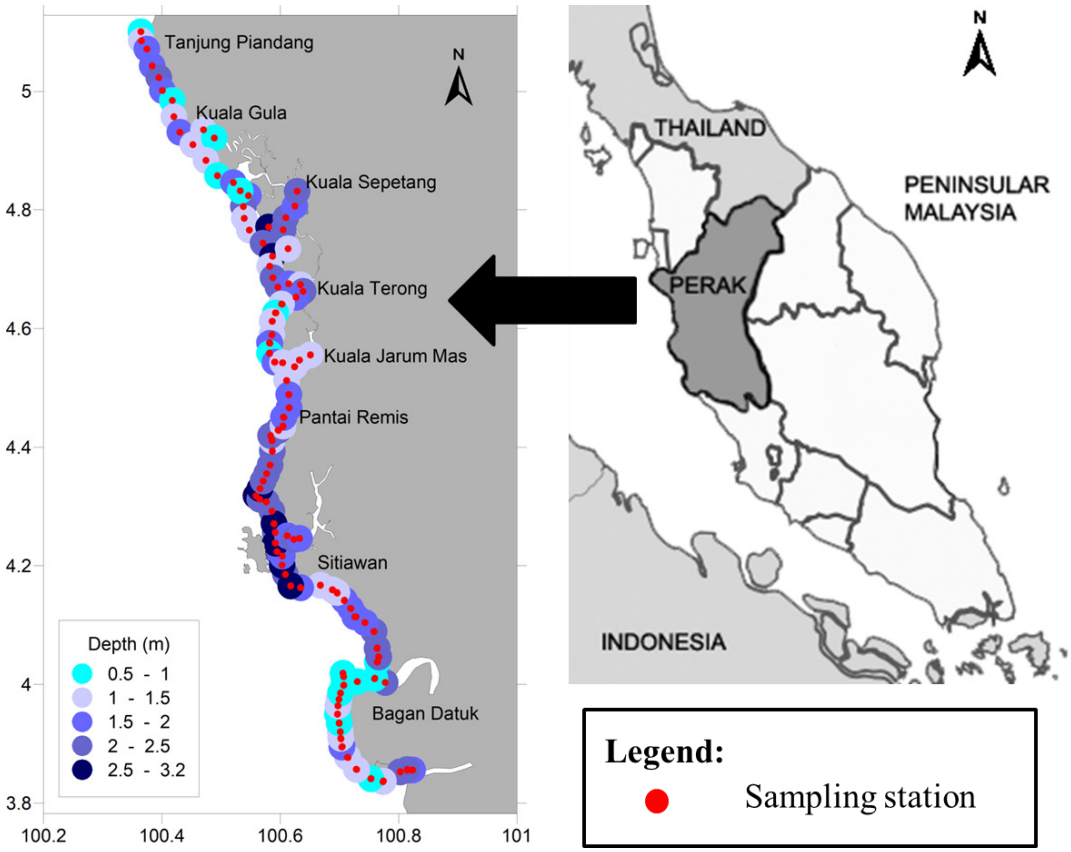

Figure 2. The sampling locations in the coastal waters of Perak from the study conducted on 20-23 August – 22 November 2019 and the second session on 25 August – 18 September 2020. A total of 107 stations were sampled in this study

Results

Asiatic hard clam (M. meretrix)

The Asiatic hard clam (*M. meretrix*) is a natural fishery resource but can only be found in certain locations in the Perak coastal water. The main area for the Asiatic hard clam was at the sandbar area of Tanjung Beras Basah near the river mouth of Sungai Perak. There was also another population of Asiatic hard clam near the vicinity of Kampung Kayan, Lekir.

The average size of Asiatic hard clam sampled during the study was 37.6±1.8 mm (±SE) and ranged from 18 to 52 mm. However, the densities of Asiatic hard clam at the sandbar area of Tanjung Beras Basah ranged from 458 to 3,551 kg.km-2 (Figure 3A). While the density at Kampung Kayan was estimated to be at 745 kg.km-2. At the area between Tanjung Piandang to Kuala Terong, the Asiatic hard clam can be found near Kuala Larut at the south of Pulau Sangga Besar. The densities of Asiatic hard clam at that area ranged from 349 – 806 kg.km-2. No Asiatic hard clam can be found elsewhere in the study area.

Inequivalve ark (A. inaequivalvis)

The inequivalve ark (*A. inaequivalvis*) or locally known as 'kerang bulu' is a naturally occurring species commonly found in the same habitat as the blood cockle (*T. granosa*). The species (*A. inaequivalvis*) is not a locally cultured species but it has limited commercial value and has demand in the exotic seafood market. The results from this study indicated three main areas where the inequivalve ark can be found which were the Kuala Kurau-Kuala Gula area, Pulau Pasir Hitam and Pantai Remis-Segari area (Figure 3B). Other areas with lesser densities of the inequivalve ark populations were Pulau Terong and Sungai Tiang.

The average size of inequivalve ark sampled during the study was 36.1±1.0 mm (±SE) and ranged from 18 to 49 mm. The densities of inequivalve ark populations ranged from 49 to 20,762 kg.km-2. The highest density of inequivalve ark was found at Pantai Remis (20,762 kg.km-2), followed by Pulau Pasir Hitam (2,764 kg.km-2). The densities of inequivalve ark at Pulau Terong and Sungai Tiang were both 54 and 74 kg.km-2 respectively.

Olive whelk (N. olivaceus)

The olive whelk (*N. olivaceus*) is a type of gastropod which can be found near the mangrove habitat in Perak waters. It is a sought after by the exotic seafood market and is usually a by-catch in fisheries or collected during blood cockle harvesting activities. The results from this study indicated the distribution of olive whelk was mainly found in the northern region of Tanjung Kupang, at the river mouth of Sungai Perak (density of 2,037 kg.km-2) and at the river mouth of Sungai Bernam at Tanjung Beras Basah (density of 1,113 kg.km-2). The area near Kuala Jarum Mas also had a sizeable olive whelk population (densities ranging from 185 to 824 kg.km-2). The average density of olive whelk was 387±97 kg.km-2 (±SE) and ranged from 14 to 2,037 kg.km-2 (Figure 3C). The average size of olive whelk was 20.4±0.5 mm (±SE) and ranged from 9 to 38 mm.

In general, the distribution of olive whelk populations were mainly found in the southern region of Perak waters, from the river mouth of Sungai Bernam to Manjung district. Higher densities of olive whelk were found in Bagan Datuk compared to Lekir or Manjung areas. On the contrary, no olive whelk sample was collected during sampling in the Tanjung Piandang to Lumut area, except for two locations at Kuala Jarum Mas.

Tiger moon snail (N. tigrina)

The tiger moon snail (*N. tigrina*) is another gastropod species with minor commercial value which can be found in several areas in Perak waters. This gastropod species is also a by-catch in fisheries or collected during blood cockle harvesting activities. The results from this study indicated the highest concentration of tiger moon shell was at Kampung Kayan (density of 3,458 kg.km-2) but also could be found elsewhere especially in the area from Sungai Bernam river mouth to the vicinity of Kampung Pasir Panjang in Sitiawan (Figure 3D). The average density of tiger moon snail was 313±129 kg.km-2 (±SE) and ranged from 12 to 3,458 kg.km-2. The average size of tiger moon snail collected in this study was14.1±0.3 mm (±SE) and ranged from 6 to 33 mm.

Aside from the southern waters of Perak, tiger moon snail can also be found in other locations such as Pantai Remis, Kuala Jarum Mas, Kuala Terong, Kuala Larut and Pulau Sangga Kecil but in smaller populations. Tiger moon snails are natural predators of cultured blood cockle (*T. granosa*) and usually can be found in cockle culture areas mentioned above. In relation, high concentration of tiger moon snails in such areas will pose a problem to the blood cockle culture practice as harvest would dwindle and increased maintenance effort by culturists to remove the predatory snails from their culture plots. Common practice by blood cockle culturist to remove the predatory snails is by mean of periodically dredging the culture plots with a long dragged scoop and physically collecting the snails (and other debris) from the dredge basket.

Murex Shell (Murex spp*.)*

The murex shell (*Murex* spp.) is a type of gastropod which are consumed locally and has good commercial value. It is also known locally as 'siput duri' and are commonly found in the exotic seafood restaurants. This study indicated that they can be found in several areas in the northern region of Perak waters. From this study, the murex shell was obtained in 39 locations (Figure 3E). The average density of murex shell was 228±28 kg.km-2 (±SE) and ranged from 39 to 722 kg.km-2. The average size of murex shell collected during this study was 59.5±1.4 mm (±SE) and ranged from 22 to 99 mm. The highest density of murex shell was found at the coastal area of Pulau Pasir Hitam (722 kg.km-2).

In term of distribution, the murex shells were commonly found near mangrove habitat such as the area from Larut Matang to Pantai Remis. There was also presence of murex shells in lesser densities (0 - 250 kg.km-2) in the area from Tanjung Piandang to Kuala Gula. Other areas such as Kampung Sungai Tiram (Lekir) and Damai Laut (Lumut) also had low densities of murex shell (56 and 6868 kg.km-2 respectively). However, there were no murex shell samples collected in during sampling at the southern region from Hutan Melintang to Bagan Datuk area.

Murex shells are also natural predators of cultured blood cockle (*T. granosa*) and usually can be found in cockle culture areas. Therefore, high concentration of murex shells in blood cockle culture area will pose a problem to the blood cockle culturists as more effort would be needed to remove the predatory snails from the culture plots or they would suffer decreasing blood cockle harvest.

Spiral melongena (Pugilina sp.)

The spiral melongena (*Pugilina* sp.) is a type of gastropod which are consumed locally and has good commercial value. It is also known locally as 'siput unam' and was found in 21 locations in Perak during this study (Figure 3F). The average density of spiral melongena in the study area was 629±125 kg.km-2 (±SE) and ranged from 95 to 2,320 kg.km-2. The average size of the spiral melongena caught during this study was 58.5±2.7 mm (±SE) and ranged from 7 to 78 mm. The highest density of

that gastropod could be found in the waters of Pulau Pasir Hitam (2,320 kg.km-2).

The spiral melongena are commonly found near mangrove habitat especially from Larut Matang to Pantai Remis area. Beside the main population of spiral melongena at Pulau Pasir Hitam, there was also high density of spiral melongena at Pulau Kalumpong (near Kuala Gula). Other area such as Lumut, Pantai Remis, Kuala Jarum Mas, Kuala Terong, Kuala Larut, Tanjung Piandang and Teluk Rubiah also had spiral melongena populations but in lesser densities (95 - 1000 kg.km-2). However, from Lekir southward there was no spiral melongena presence during the course of this study.

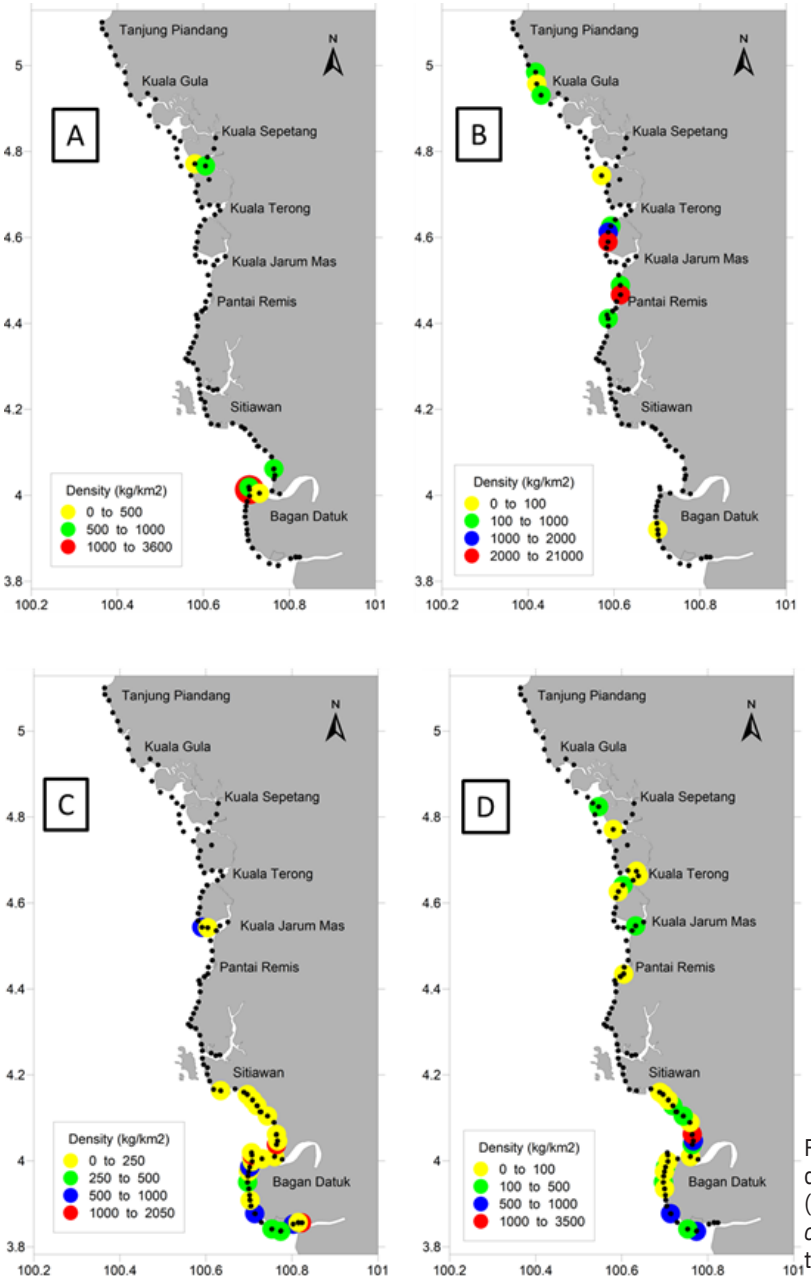

Figure 3 (A-D). Distribution and density (kg.km-2) of *M. meretrix* (A), *A. inaequivalvis* (B), *N. olivaceus* (C) *and N. tigrina* (D) in the coastal waters of Perak

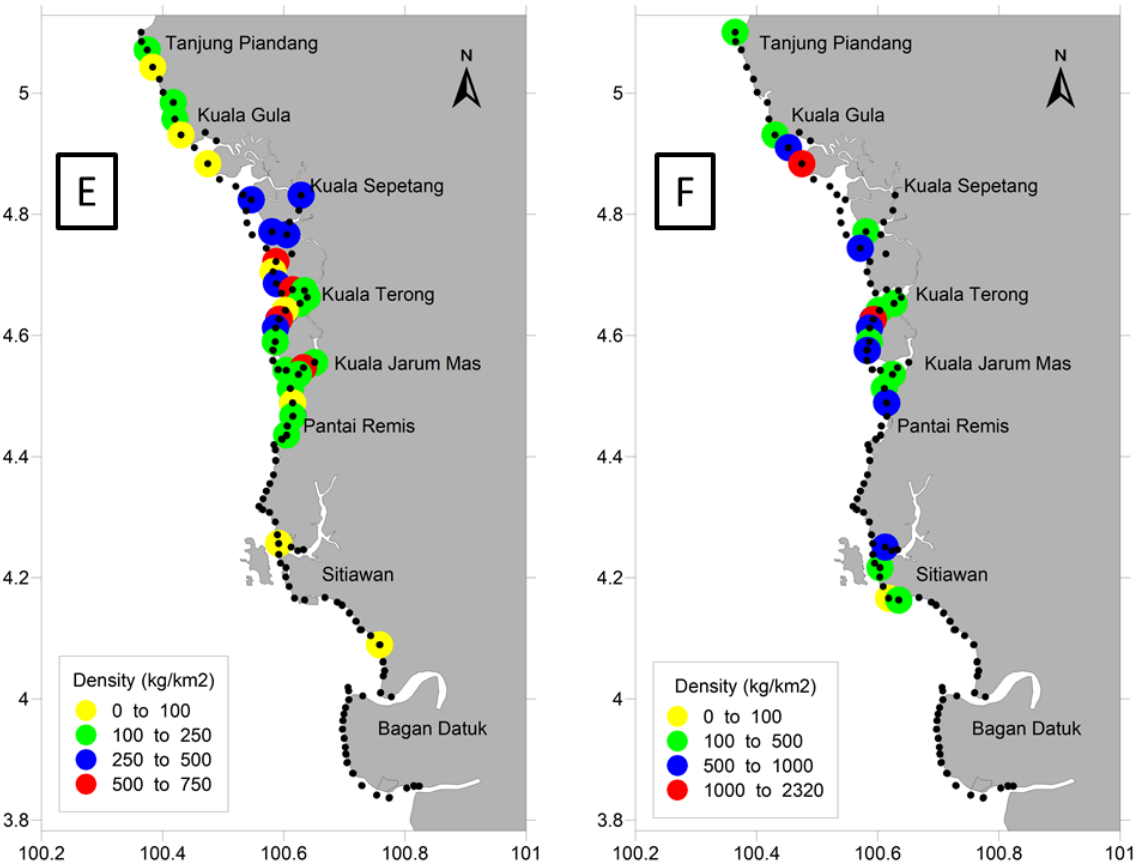

Figure 3 (E-F). Distribution and density (kg.km-2) of *Murex* spp. and *Pugilina* sp. in the coastal waters of Perak

Discussion

Community-based management of bivalve resource

(M. meretrix The Asiatic hard clams) mainly occur in two areas, namely the river mouth of Sungai Perak and to a lesser degree near the coastal area at Kuala Sepetang. Near the river mouth of Sungai *M. meretrix* Perak, most of the population concentrated on a sand dune area at Tanjung Beras Basah. This site has been consistently harvested by local fishermen using two methods namely collection of Asiatic hard clams by hand during low tide or using the long-dragged scoop. However, the second method can be operated in longer duration as it is not limited to the short time frame when the tide is low enough for the collectors to walk to the clam site while the number of clams harvested is also greater than by hand collection. The discrepancy in catch volumes between the two methods has created some frictions between the groups of people who collect the clams by hand with those that operated the long-dragged scoops.

To better manage the Asiatic hard clam resource in the Sungai Perak area, some control measures may be required such as bag limit, clam conservation zone and harvesting season. The implementation of such control measures would require further consultations by fisheries managers with various fisheries communities in the area, particularly to secure their cooperation and participation in management and sustainable utilization of the available Asiatic hard clam resource. A local community-based management group to manage the Asiatic hard clam resource would be ideal in

such situation and fisheries managers as well as local scientists could also participate in sharing ideas and information regarding the sustainable management of the clam resource (*cf.* Jarina et al., 2018).

Gastropods presence in blood cockle farms

The distribution of gastropods such as *N. tigrina, Murex* spp. and *Pugilina* sp. near the mangrove habitat area are closely related to the availability of prey organisms such as bivalve in the area. Aside from the cultured blood cockle, there are also other natural occurring bivalve species such as nut clams (*Nucula* sp.) and mussels which provide sufficient prey organisms for the predatory gastropods. However, the excessive presence of such predatory animals are problems to the aquaculturists who culture blood cockle in Perak waters.

There are 104 blood cockle culturists in Perak and the total culture area was about 2,238 hectare (DOFM, 2020). In year 2020, Perak aquaculturists produced 15,130 metric ton or nearly 74% of all cultured mollusks in Malaysia (DOFM, 2020). However, out of this figure, 14,834 metric ton was from the production of blood cockle (DOFM, 2020). The presence of large number of predatory gastropods in blood cockle culture area will pose a problem to the blood cockle culturists as more effort would be needed to remove the predatory snails from the culture plots or they would suffer decreasing blood cockle harvest.

Nonetheless, gastropods such as murex shells, tiger moon snails, spiral melongenas and even olive whelks are locally consumed and are sold in the exotic seafood market. Together with the Asiatic hard clams and inequivalve arks, these mollusks have good commercial values even if they are not normally considered aquaculture species and supply may be seasonal. Through proper harvesting and distribution mechanisms (to the market), the gastropods can contribute to the blood cockle culturists side revenues as they are collected during periodical dredging of the farm's bottom sediment to redistribute the blood cockle in culture and to remove debris and predators.

Horse mussel (Modiolus sp.*) colony near blood cockle culture activities*

In the course of this study the presence of horse mussel (*Modiolus* sp.) colony was detected in the vicinity of Pulau Sangga Besar in the Larut Matang area. The density of the horse mussel in the area may exceed well over 30 metric ton.km-2 and the location of the colony was near to the blood cockle area. This situation may present a potential problem to the blood cockle population in the Pulau Sangga Besar area. The horse mussel species produce very dense byssal threads formation and normally the colony can form a large span of matting that cover the sea bottom (Yap, 1978). Such matting inhibits the growth of other bivalve populations (such as blood cockle) and can alter the pattern of sediment deposition (Carranza et al., 2009).

The bioinvasion of foreign species in bivalve aquaculture elsewhere has reduced the production of cultured species and burdened the industry financially, which need to remove and eliminate the invasive species from their culture area (Vallejo et al., 2017; Lim et al., 2018; Biju Kumar et al., 2019). This condition will threaten the cockle farming activities in the area and efforts must be made to remove the horse mussel colony from the blood cockle culture area by means of physical extraction and disposal on land. However, such exercise requires large manpower and is very time consuming but is essential to protect the blood cockle culture area.

The inability to control the horse mussel population will limit available culture area for blood cockle as horse mussel species can easily outcompete the other bivalve species in term of spatial colonization (*cf.* Sanpanich and Wells, 2019). The disposal of horse mussel back into the sea in other

area is strictly not advisable as this action will inadvertently spread the horse mussel population to colonize other coastal area. Alternatively, the horse mussel could be explored as a new food source for the public or processed into aquaculture feeds as practiced in the Philippines (Napata and Andalecio, 2011). The protein sourced from such non-commercial bivalve species such as horse mussels or nut clams should be further studied by feed nutrition specialists and formulate ways to effectively harvest and process the abundant bivalve resources for use in aquaculture feed formulation.

Conclusion

The main area for the Asiatic hard clam was at the sandbar area of Tanjung Beras Basah near the river mouth of Sungai Perak. The density of Asiatic hard clam there ranged between 458 to 3,551 kg.km-2. To better manage the Asiatic hard clam resource in the that area, some control measures may be required such as bag limit, clam conservation zone and harvesting season. The formation of a local community-based management group is suggested to better manage the Asiatic hard clam resource available and tackle any conflict arising from the different groups of stakeholders.

As for the inequivalve ark, this species can be found at three main areas namely Kuala Kurau-Kuala Gula, Pulau Pasir Hitam and Pantai Remis-Segari area while the distribution of olive whelk was mainly found in the northern region of Tanjung Kupang, at the river mouth of Sungai Perak and at the river mouth of Sungai Bernam at Tanjung Beras Basah. The distribution of tiger moon snail, murex shell and spiral melongena are related to the mangrove habitat and can be found mainly at the northern Perak region from Tanjung Piandang to Pantai Remis.

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Coral Reef Fishes Biomass Estimation Using Visual Observation at Pulau Sembilan Archipelago

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Abstract: Pulau Sembilan archipelago has been proposed to be the first Marine Park in Perak. We conducted a study of coral reef fishes in the island cluster to determine the reef fish's status. This study was conducted using the SCUBA survey with an area of 250 square meters each. Small pelagic fish species and coral reef indicator species were recorded during our study. Cryptic and small fishes such as goby and blennies were excluded. Each transect was placed parallel to the shoreline at a five-meter depth. Divers swam along the transect and count the number of fishes, fish species and estimated the fish size. The number of damselfish topped at all study locations with a frequency of over 76% followed by *Pterocaesio chrysozona* (11.9%) while other fishes were below 3%. Commercially valuable fishes for human consumption such as *Caesio teres* and *Cephalopolis formosa* were found at 1.33% and 1.29% respectively. The total fish biomass was calculated at 740 kg/ha. Pulau Payung Kecil (PPK) had the highest species diversity compared to other sites. Fishes from the family Sygnatidae like seahorses and pipefish were also observed during the sampling in the reef habitat. The highest H' diversity index was at PPK (0.5) followed by PP (0.4), PA (0.3) and PSK (0.3). The lowest value was at PR1 (0.1). Pulau Payung (PP) had the highest number of fish species compared to other islands. We conclude that this area needs attention for coral fishes conservation and the proposed marine park will help protect the marine resources.

Keywords: Pulau Sembilan, Fish Species, Shannon Weiner Index, Biomass, Commercial Fish

Abstrak: Kepulauan Pulau Sembilan telah dicadangkan untuk menjadi Taman Laut pertama di Perak. Kami menjalankan kajian ikan terumbu karang di gugusan pulau untuk menentukan status ikan terumbu. Kajian ini dijalankan menggunakan tinjauan SCUBA dengan keluasan 250 meter persegi setiap satu. Spesies ikan pelagik kecil dan spesies penunjuk terumbu karang direkod semasa kajian. Ikan tersembunyi dan kecil seperti goby dan blennies dikecualikan. Setiap transek diletakkan selari dengan garis pantai pada kedalaman lima meter. Penyelam berenang di sepanjang transek dan mengira bilangan ikan, spesies ikan dan menganggarkan saiz ikan. Bilangan ikan damsel mendahului di semua lokasi kajian dengan kekerapan melebihi 76% diikuti oleh *Pterocaesio chrysozona* (11.9%) manakala ikan lain berada di bawah 3%. Ikan yang bernilai komersial untuk kegunaan manusia seperti *Caesio teres* dan *Cephalopolis formosa* didapati masing-masing pada 1.33% dan 1.29%. Jumlah biojisim ikan dikira pada 740 kg/ha. Pulau Payung Kecil (PPK) mempunyai kepelbagaian spesies yang paling tinggi berbanding tapak lain. Ikan daripada keluarga Sygnatidae seperti kuda laut dan ikan paip turut diperhatikan semasa persampelan di habitat terumbu. Indeks kepelbagaian H' tertinggi ialah pada PPK (0.5) diikuti oleh PP (0.4), PA (0.3) dan PSK (0.3). Nilai terendah ialah pada PR1 (0.1). Pulau Payung (PP) mempunyai bilangan spesies ikan tertinggi berbanding pulau-pulau lain. Kami membuat kesimpulan bahawa kawasan ini memerlukan perhatian untuk pemuliharaan ikan karang dan cadangan taman laut akan membantu melindungi sumber marin.

Introduction

Pulau Sembilan Archipelago is surrounded by deep trenches and fast-flowing current limiting the underwater visibility. The reefs are usually only dived during neap tide and dry season. Selection of diving location and time are crucial when organizing a dive trip there. Our study was conducted in September 2020 to gather the information needed to support the proposal to gazette this archipelago as a Marine Park for the state of Perak.

Reef fishes are important as one of the coral reef condition indicators. The fishes contribute to the fish catches and landing consequently ensuring the balance of the ecosystem food chain. A survey on the fish species composition, density and frequency is a way to categorize the condition of the reef (Schmitt et al., 2002). The information obtained support the management and conservation of marine ecosystems.

The methods for conducting coral reef fish biomass studies varies from catching using nets, traps or harpoon (Mukhashen, 1992). Recently underwater photography or video filming were also introduced. These methods do not threaten the fish resources and are commonly used in marine parks or conservation areas (Nasir et al., 2017). Experience observers also used visual counts (Allen, 1999). Visual survey technique requires a skilled researcher, able to identify reef fishes quickly and also make fish size estimates in situ (Yusuf et al., 2001; Nasir et al., 2017).

Chew et al. (2008) identified 24 families of fish larvae at Pulau Sembilan mainly from the family Carangidae, Engraulidae, Gobiidae and Signatidae. However, the density was lower near the islands compared to the open water further away (Chew et al., 2008). On the other hand, 72 species of coral reef fishes have been recorded around nearby Pangkor Island and mainly from the family Pomacentridae, wrasses (Labridae), goby (Gobiidae) and grouper (Serranidae) (Chew et al., 2008). As a comparison, researchers from Indonesia recorded a total of 108 species of coral fishes around Ternate Island (Utama et al., 2019).

This study was designed to find out the fish biomass and diversity between the islands in the archipelago using visual observation. We will also determine the number and species of commercial fish available. Next, by using the fish species and number, the diversity index of the reef fishes at the archipelago will be determined.

Materials and Methods

Pulau Sembilan archipelago consists of 12 islands including rocky outcrops. The islands are unpopulated except Pulau Lalang which has a temporary station for the Perak State Park ranger (Figure 1). Sixteen sites were selected for this survey covering all the islands (Table 1). We surveyed using underwater visual observation focusing on the commercial and coral related fishes (Schmitt et al. 2002; Hodgson et al. 2006). The fishes were categorized into; a) commercial demersal and pelagic species, b) coral-related fishes and c) other valuable and conspicuous fishes. In this study, small and cryptic fishes were not recorded. These include goby fish and blennies found in this area. Species were identified using references (Allen, 1999).

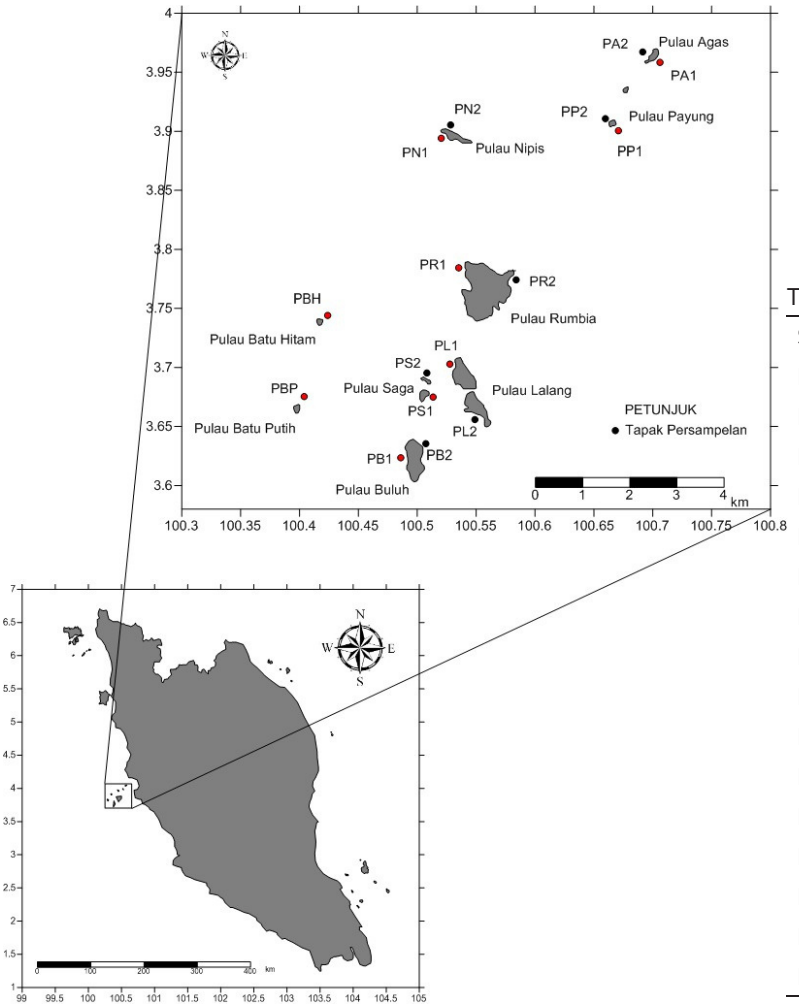

Table 1 Study sites

Site	Pulau
PL1	Pulau Lalang 1
PR1	Pulau Rumbia 1
PL2	Pulau Lalang 2
PBP	Pulau Batu Putih
PB1	Pulau Buluh 1
PS1	Pulau Saga 1
PB2	Pulau Buluh 2
PN1	Pulau Nipis 1
PA	Pulau Agas
PP	Pulau Payung
PL 2	Pulau Lalang 2(2)
PR 2	Pulau Rumbia 2
PSK	Pulau Saga Kecil
PN 2	Pulau Nipis 2
PPK	Pulau Payung Kecil
PA2	Pulau Agas 2

Figure 1. Map of Pulau Sembilan Archipelago

Observations were made using transects as a guide for the survey area covered. The transect length used was 50 m and 5 m wide with an area of 250 m2. Observations were made along the transect without dividing it into several parts due to the limited observation distance and the same fish appeared to be repeated over the transect several times. A diver swims along the transect observing the fishes and stop a few times to write down the observations.

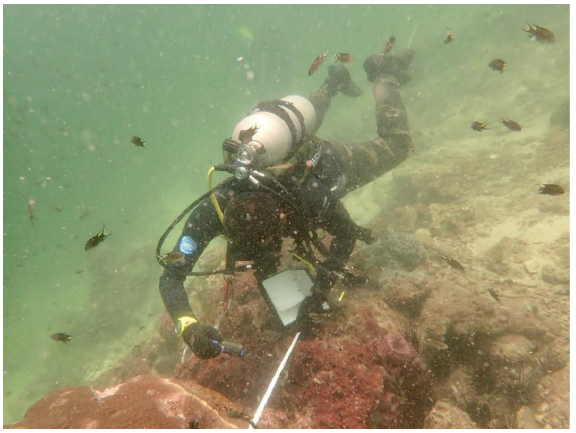


Figure 2. Survey method and the visibility of the water

The initial dive determined the number of common fish species as a guide for the survey list preparation. During the survey, the number of fish was counted and the size of each species was estimated using a measuring stick. The average size of each fish species was recorded for biomass calculation (Kulbicki et al., 2005).

The number of fishes was used to calculate the biodiversity index for each site. Shannon Weiner Index, Evenness and Simpson Reciprocal Index (Zar, 2010). The indices were plotted using excel software and were used for discussion in this report.

Results

Twenty-seven fish species were recorded during this study (Table 2). Damsel fishes were dominant at every site followed by *Pterocaesio* sp. The damsels were related to the corals, especially the branching *Acropora* sp.

Table 2 Fish species and observation percentages.

	Species% observation	
1	*Neopomacentrus azysron*	76.4
2	*Pterocaesio chrysozona*	4
3	*Neoglyphidodon nigroris*	11.8
4	*Caesio teres*	5
5	*Cephalopolis formosa*	2.79
6	*Siganus canaliculatus*	1.33
7	*Amphiprion sandaracinos*	1.29
8	*Scolopsis ciliatus*	0.88
9	*Scolopsis vosmeri*	0.81
10	*Chaetodon octofasciatus*	0.81
11	*Halichoeres nebulosus*	0.64
12	*Siganus javus*	0.54
		0.51
		0.45

1	*Lutjanus vitta*	0.3
3	*Scolopsis trilineatus*	8
1	*Thalassoma lunare*	0.3
4	*Epinephelus boenack*	5
1	*Pempheris*	0.1
5	*Chaetodon colare*	8
1	*Zanclus cornutus*	0.1
6	*Pomacanthus annularis*	6
1	*Scarus*	0.1
7	*Lutjanus lemniscatus (juv)*	6
1	*Abudefduf vaigiensis*	0.1
8	*Gnathanodon speciosus*	2
1	*Heniochus acuminatus*	0.1
9	*Sargocentron*	0
2	*Diodon*	0.0
0		5
2		0.0
1		4
2		0.0
2		3
3		0.0
2		4
2		0.0
5		2
		0.0
		2
		0.0
7		2

Neopomacentrus azysron., *Pterocaesio chrysozona*. *Neoglyphidodon nigroris, Caesio teres* and *Cephalopolis formosa* were the most abundant fishes (Figure 2). The diversity of fishes in the Pulau Sembilan archipelago was at an H' average of 0.19 (Figure 3). In addition, only 27 species were among the main and most common species that can be seen around the transects. In terms of number, damselfish topped at all study locations with the frequency of observations recorded exceeding 76%. Pterocaesio fish followed this by 11.9%. The percentage of findings of other fishes were below 3%. Commercially valuable fishes for human consumption such as *Caesio teres* and *Cephalopolis formosa* were found with rates of 1.33% and 1.29%. Two types of groupers recorded were *Cephalopolis formosa* and *Epinephalus boenack*. However, only *C. formosa* was found to be larger than 15 cm. The murky water conditions and bad weather affected the survey activities. Heavy rain and high cloud cover made observation difficult.

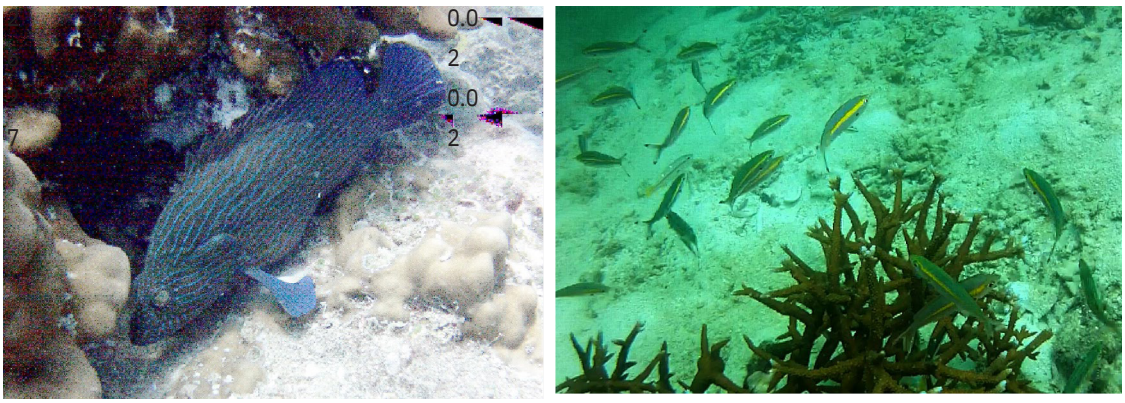

Figure 2. Two of the common fish species *Cephalopolis formosa* and *Pterocaesio sp*.

Sea horses and Pipefishes from the Sygnathidae species were not recorded during the study as they were not found around the transect. Observations outside the transect found that there were more than 3 species of seahorses here. There were also several species of pipefish fish from the same

family.

The blennies and gobies were not recorded because the numbers were high, small and cryptic, which made it difficult to estimate quickly. This species was also not a target species during this study.

The highest number of fish recorded in PP with the number of fish of 1.6872 fish/m2. This is followed by PA with the number of fish of 1.6696 fish/ m2 and PPK with the number of fish of 1.3704 fish/ m2. Throughout the study, it was found that the number of damsels and Pterocaesio fish outnumbered other fishes by more than 10 times. The calculation of the number of fish can be done with two conditions, namely without damsel and pterocaesio fishes or with both species combined. The number of fishes counted without damsel and Pterocaesio showed the highest number of study locations were PPK and PS1. While the calculation of the number of fish including the two species showed the top locations were at PP and PPK.

The total number of fish counted during the study was 12,742 for the whole survey trip. The average number of fish per location was 0.16 individuals/m2. The largest number of fish recorded were damselfish.

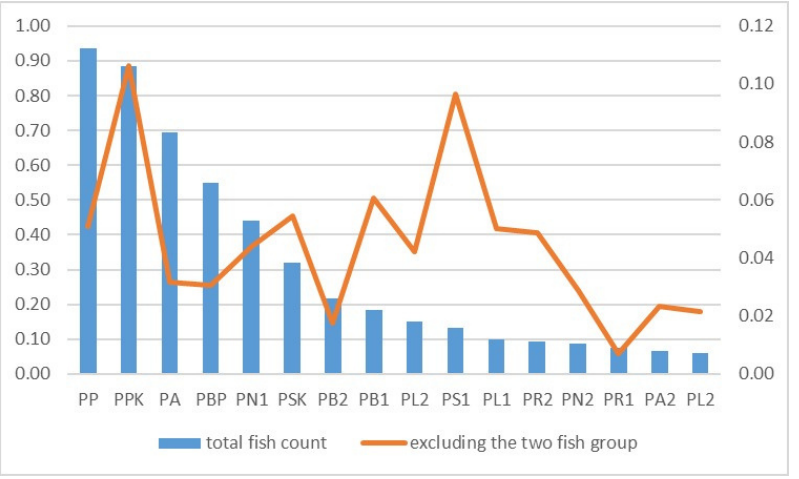

Figure 3. The total fish count in the blue bar and excluding damsel and pterocaesio fish (orange line)

Fish biomass

The biomass of fish during the study was 0.018 kg /m3. The average biomass per study location was 0.004 kg/m3 and 0.006 kg/m3 for trip 1 and trip 2. The fish biomass recorded during the study at the study location during the first week of sampling was higher than the second week. This may be due to several factors such as the number of transects, water clarity and weather during the study. Overall, the highest fish biomass was recorded in three locations namely PPK, PB2, and PP. PPK apart from having a high diversity of species also provides a high amount of biomass compared to other places.

Diversity Index

The highest H 'index diversity was at PPK (0.5) followed by PP (0.4), PA (0.3) and PSK (0.3). The lowest value was at PR1 (0.1) (Figure 2). The highest Simpson Reciprocal Index were at PL1 and PS1 this index indicates a stable habitat and has a high niche diversity (Figure 2). This

reduces competition in habitat utilization as well as provides opportunities for more species to live together. In contrast, a low index indicates unstable habitat, low niche diversity and high competition for places

The number of fishes exceeding 15 cm body length

Commercially useful species on the reefs of Pulau Sembilan were *C. formosus, Caesio teres Lutjanus sp* and. However, the number of commercial-sized fish was between 21.7 and 16.8 per location. PS 1, PL 2 and PA2 recorded larger fish individuals compared to other locations (Figure 2). *Cephalopolis formosus, Caesio teres, Pterocaesio digramma* larger than 15 cm were found in PS 1. *Cephalopolis formosus* and *Caesio teres* were larger than 15 cm in PL 2.

Discussion

The number of fish species during this study was low (27 species) compared to other studies conducted around Pulau Sembilan and Pangkor. This was due to the limited target fishes observed. Yusuf, et al., (2009) reported 75 fish species during their survey. Several species of small and cryptic fishes like gobi and blennies were not recorded. Seahorses species were not found along the transect however there were several seahorse species found outside the transect during randomized observations. The coral reef of the Pulau Sembilan archipelago was abundant in seahorses and pipefish species (Lim & Chong, 2015). In addition, murky water conditions with low observation distance and water currents prevented better observations. Fish from the snapper species were rare and only small individuals from the species *L. biguttatus and L. vitta* were observed.

The overall fish biomass from both study trips was 0.074 kg/m2. Mukhashen (1992) reported high species diversity in their study on an Indonesian reef area with the biomass of fish caught using fishing method recorded at 0.61kg/standard catch with the main species consisting of *Lutjanus lutjanus* (Big eye snapper) and *Pterocaesio chrysozona* (gold band fusilier). Nasir et al., (2017) and Utama et al., (2019) reported biomass between 0.0060 - 0.0116 kg/m2 and 0.0042 kg/m2 in a study conducted on the coral reefs of Batee Island, Indonesia.

Table 3. Biomass of this study compared to studies elsewhere

Studies	Coral reef of Pulau Batee, Pulau Sembilan, Perak Indonesia (Nasir et al., (2017)	(this study 2020)	Ternate Island, Indonesia (Utama et al., 2019)
Fish Biomass	0.0060 - 0.0116 kg/m2	0.074 kg/m3	0.0042 kg/m2

More than 12,000 fish were counted during this study in the waters of Pulau Sembilan. The biomass exceeded the reported biomass in other areas by other researchers for example Nasir et al., (2017). The most numerous individuals found were fishes from the damsel group. Apart from damsel fish, other fishes present in the transect were semipelagic fishes such as yellow and blue- backed fusilier (*Caesio teres*) and gold band fusilier fish (*Pterocaesio chrysozona*). Ranking results differed if the total number of fishes was calculated without the two main species namely damsel and pterocaesio. The results of this study showed that these reefs were dominated by planktivory fishes such as damsel and fusilier which contribute to more than 50% of the recorded biomass. Locations influenced by the number of damsels may indicate areas that are important as nursery areas or feeding areas of predatory fish.

Large and commercially valuable fishes observed were grouper *Cephalopolis formosus*, *Caesio terra* and *Gnathanodon speciosus.* During this study, the largest fish recorded was *Scarus* sp.

which was larger than 30 cm in length. This may be due to low visibility and fishes did not approach researchers using SCUBA equipment.

The Simpson index values showed that Pulau Payung Kecil (PPK) was the highest compared to other places. This was followed by Pulau Payung and Pulau Agas. This may be due to the higher diversity of niches that accommodated various types of fishes with lesser competition for habitat space.

Conclusion

Our study showed that Pulau Sembilan has high fish biomass compared to other places referred to. The fish biomass recorded was at 740 kg/ha. Pulau Payung Kecil and Pulau Payung were high in fish species diversity compared to other islands within this archipelago, which may be due to the coral reef complexity. A diverse group of fishes inhibits the coral reef of the Pulau Sembilan archipelago and needs protection that is crucial to protect the fishery resources.

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Molecular Characterisation of Segment 3 Tilapia Lake Virus (TiLV) Isolated from Different Localities in Malaysia

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Abstract: Malaysia has declared TiLV positive in 2017 and following this OIE declaration, research and monitoring have been in place. This paper focuses on the molecular characterisation of the TiLV isolated from tilapia fish at different regions in Malaysia, during epidemiological studies and outbreak cases. The samples were collected between 2017 and 2019. The study aimed to identify if there are any molecular differences between the isolates, particularly on segment 3. Organs samples such as liver, kidney and spleen were pooled into viral transport media, processed, and inoculated into E-11 cell line. The inoculated cells were observed for any cytopathic effect (CPE) formation for at least 3 passages, before being harvested and confirmed with semi-nested RT-PCR. From 62 samples inoculated into cell culture, 33 samples (53.2%) were positive for CPE and 22 samples were selected and sent for sequencing. The sequences went through multiple nucleotide sequence alignments with the database in GenBank in the National Centre for Biotechnology Information (NCBI), and a phylogenetic tree was constructed by using MEGA 7 applying a maximum likelihood bootstrap analysis (1000 replication). The sequence similarities showed that segment 3 of all TiLV isolated from tilapia fish in this study had at least 98% similar to TiLV isolated from Thailand (accession number: KY381578.1).

Keywords: tilapia, TiLV, molecular characterisation, phylogenetic tree

Abstrak: Malaysia telah mengisytiharkan TiLV positif kepada OIE pada tahun 2017 dan setelah pengisytiharan OIE ini, penyelidikan dan pemantauan telah dilaksanakan. Artikel ini memfokuskan kepada pencirian molekul TiLV yang diasingkan dari ikan tilapia di beberapa kawasan di Malaysia, semasa epidemiologi dan wabak penyakit. Kajian ini bertujuan untuk mengenal pasti sama ada terdapat perbezaan molekul pada segmen 3 antara isolat TiLV yang dipencilkan. Sampel yang dikumpulkan adalah antara tahun 2017 dan 2019. Sampel organ seperti hati, ginjal dan limpa digabungkan dalam media pengangkutan virus, diproses dan diinokulasi ke dalam sel E-11. Sel-sel yang diinokulasi diperhatikan untuk sebarang pembentukan kesan sitopatik (CPE) untuk sekurang- kurangnya 3 *sub-passage*, sebelum dituai dan disahkan dengan *semi-nested* RT-PCR. Dari 62 sampel yang diinokulasi ke dalam kultur sel, 33 sampel (53.2%) positif untuk CPE dan 22 sampel dipilih untuk dibuat penjujukan cDNA. Hasil jujukan cDNA dibandingkan dengan pangkalan data di GenBank di Pusat Maklumat Nasional Bioteknologi (NCBI) dan pohon filogenetik dibentuk menggunakan perisisan MEGA 7 melalui analisis bootstrap kemungkinan maksima (1000 replikasi). Hasil kajian menunjukkan bahawa segmen 3 dari semua TiLV yang diasingkan dari ikan tilapia dalam kajian ini mempunyai sekurang-kurangnya 98% persamaan dengan TiLV yang diasingkan dari Thailand (nombor akses: KY381578.1).

Introduction

Tilapia is popular species for aquaculture due to its fast growers, efficient food converters, omnivorous diet, high-density aquaculture and relative disease resistance. It also serves as an important protein source, especially in developing countries (FAO 2016). Tilapia have been a major protein source for the poor countries, and the emergence of TiLV will have a huge impact on the industries locally and globally. The disease could affect US$ 7.5 billion a year in production to major producing countries including China, the Philippines, Thailand, Indonesia, Lao PDR and Bangladesh (Network of Aquaculture Centres in Asia-Pacific 2017).

Since 2009, substantial mortality of tilapia have occurred in Israel and Ecuador, and in 2014, a virus was identified as the cause of this death (Eyngor et al., 2014)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. Since then, TiLV outbreak has been reported to be present on three continents: Asia, Africa and America. Countries affected by TiLV include Colombia (Kembou Tsofack et al., 2017), Ecuador (Ferguson et al., 2014; Kembou Tsofack et al., 2017), Peru (Pulido et al., 2019), Israel (Eyngor et al., 2014), Egypt (Fathi et al., 2017), Uganda and Tanzania (Mugimba et al., 2018), Malaysia (Abdullah et al. 2018; Amal et al., 2018), Thailand (H. T. Dong et al., 2017; Surachetpong et al., 2017), Indonesia (Koesharyani et al., 2018), India (Behera et al. 2018), the Philippines (OIE 2017), Bangladesh (Chaput et al., 2020), United States, Mexico and Kenya (Aich et al., 2022).

TiLV virus resembles a virus in the family orthomyxoviridae, and is temporarily called tilapia lake virus (TiLV) (Eyngor et al., 2014)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. TiLV, officially known as Tilapia tilapinevirus is of the genus Tilapinevirus and family Amnoonviridae (Kuhn 2018; Siddell et al., 2019). The TiLV is an icosahedral virus and has 10-segment, negative-sense single-stranded RNA (-ssRNA) genome with total size of 10,323 kb and a diameter between 55 and 100 nm (Bacharach et al., 2016; del-Pozo et al., 2016; Eyngor et al., 2014; Ferguson et al., 2014; Surachetpong et al., 2017)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. The largest segment, segment 1, contains open reading frames with weak sequence homology to the PB1 subunit of influenza virus C. The other nine segments do not show homology to other viruses but have complementary sequences at terminals 5′ and 3’, respectively, in line with the existing genome organization in another orthomyxovirus (Bacharach et al., 2016)tolerance for high-density aquaculture, and relative disease resistance. Since 2009, tilapia aquaculture has been threatened by mass die-offs in farmed fish in Israel and Ecuador. Here we report evidence implicating a novel orthomyxo-like virus in these outbreaks. The tilapia lake virus (TiLV. *In situ* hybridization shows replication and transcription of TiLV at the site of pathology in the liver and central nervous system with disease (Bacharach et al., 2016; Eyngor et al., 2014) tolerance for high-density aquaculture, and relative disease resistance. Since 2009, tilapia aquaculture has been threatened by mass die-offs in farmed fish in Israel and Ecuador. Here we report evidence implicating a novel orthomyxo-like virus in these outbreaks. The tilapia lake virus (TiLV. Outbreaks and experimental studies showed that TiLV could be transmitted horizontally and vertically. Horizontal transmission of the virus has been confirmed through cohabitation of infected fish with clinically healthy fish (Eyngor et al., 2014; Liamnimitr et al., 2018). Vertical transmission affects mainly early developmental stages of tilapia, ie. fertilized eggs, yolk-sac larvae, fry and fingerlings (Ha Thanh Dong et al., 2015; Tattiyapong, Dachavichitlead and Surachetpong 2017; Yamkasem et al., 2019).

TiLV causes symptoms like redness on the skin, inflammation of the organs especially eyes and brain, while liver damage causes other organs failure. Examination of body tissues showed signs

of oedema, haemorrhage to leptomeninges and congestion of organs such as the kidneys, liver and spleen (Eyngor et al., 2014; Ferguson et al., 2014; Tattiyapong, Dachavichitlead and Surachetpong 2017)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. The major change is the formation of giant cells in the liver, called syncytial hepatitis (Ferguson et al., 2014). Other changes including gill pallor, exophthalmia, body discoloration (darkening), and scale protrusion and loss (Eyngor et al., 2014; Ferguson et al., 2014; Tattiyapong, Dachavichitlead and Surachetpong 2017)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. The disease can lead to high mortality, especially at juvenile stage. TiLV affected farms cultivating Nile Tilapia (*Oreochromis niloticus*), red tilapia (*Oreochromis* sp.) and hybrid tilapia (*O. niloticus* x *O. aureus*) may experience up to 90% mortality (H. T. Dong et al., 2017; Eyngor et al., 2014; Ferguson et al., 2014; Surachetpong et al., 2017). The virus is reported to only attack tilapia, but other fish that are raised together often have no sign of illness or death (Eyngor et al., 2014).

The majority of the wild tilapia, followed by barbs (Barbonymus schwanenfeldii and Barbonymus gonionotus) and other fish species like catfish and peacock bass (Cichla sp.) but at a very low rate, died in a man-made lake in the northern part of Malaysia in June 2017 (Abdullah et al., 2018). The body, operculum, ventral, and lateral fins of the tilapia were all crimson in colour, but the eyes were normal. The brains of half the fish that were dissected were fluid, squishy, or hemorrhagic. With the exception of one fish's pale liver and a somewhat congested kidney, river barb were in good physical condition. All of the tilapia samples tested positive for TiLV but negative for VNN and iridovirus, according to the PCR data. Surprisingly, the results for TiLV in river barbs that appeared healthy were also positive. Sequences from both tilapia and river barb are 96% similar to Segment 3 TiLV isolate Til42011 from Israel with Accession No. KU751816.1 after the amplicons received for sequencing were blasted in the GenBank. Understanding the genetic variants of the virus, carriers (fish that are subclinically infected), and vulnerability of other fish species to the virus are therefore necessary. In this investigation, the TiLV isolated from tilapia fish in Malaysian waters was genetically characterised.

Materials and methods

Fish samples and inoculation

Pools of liver, kidney and spleen of tilapia (*Oreochromis* spp) from Sarawak (15 fish), Perak (36 fish) and Perlis (291 fish) in 10 mL HBSS (Gibco, Thermo Scientific, Massachusetts, USA) transport medium containing 2% fetal calf serum (Gibco) and 100 µgmL−1 gentamycin-sulphate (Gibco) were processed according to methods described in Abdullah et al., (2017). 1ml of the supernatant then inoculated into E-11 cell lines (Iwamoto et al., 2000). The culture was maintained in Leibovitz's-15 (L-15, Gibco) medium supplemented with 2% foetal calf serum (FCS, PAA Laboratories) and 100 µgmL−1 gentamycin-sulphate (Gibco) and incubated at 25oC. The cells were observed daily for any cytopathic effects (CPE) formation for at least 3 passages (4-7 days/passage), before harvested. The CPE was confirmed with semi-nested reverse transcriptase–polymerase chain reaction technique (RT-PCR). 22 positive samples from the cell culture supernatant from Sarawak, Perak and Perlis were selected.

Nucleic acid extraction, RT-PCR and sequencing analysis

Total nucleic acid was extracted from the supernatant using Viral Gene-spin™ Viral DNA/RNA Extraction Kit (Intron Biotechnology, Gyeonggi-do, South Korea). Positive control for TiLV was

obtained from Dr. Thanh Ha Dong, Centex Shrimp, Mahidol University, Thailand. A set of new primers (forward: 5' TGGGCACAAGGCATCCTAC 3' and reverse 5' CACGTGCGTACTCGTTCAGT 3') targeting segment 3 of TiLV were designed using National Centre for Biotechnology Information (NCBI) software. Semi-nested RT-PCR assay for TiLV detection using total RNA extracted from the fish internal organ (liver, kidney and spleen) and cell culture supernatant as templates was performed following protocol described earlier by Dong et al., (2017). Briefly, RT-PCR was performed with reverse transcription at 45°C for 30 minutes, polymerase activation at 95°C for 60s followed by 30 cycles of denaturation at 94°C for 10s, annealing at 60°C for 10s, extension at 72°C for 30s and final extension at 72°C for 5 min, using MyTaq™ One Step RT-PCR kit (Bioline, UK) on T100 thermal cycler (BioRad, USA). The PCR products were analysed by electrophoresis in 1.5% agarose gel and stained with RedSafe™ (iNtRON, Korea). The results were viewed with bio-imaging system (Syngene, Cambridge, UK) and expected amplicon of 245bp was successfully amplified by single round amplifications.

Positive PCR products were purified using innuPREP DNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's protocol. 245bp of purified products were sent for sequencing and the nucleotide sequencing of virus were compared with the known sequences in the GenBank database Nucleotide Basic Local Alignment Search Tool (BLAST) program. Molecular phylogenetic tree analysis was generated by Maximum Likelihood method of the MEGA 7 software (Koichiro Tamura et al. 2012)the practical application of these methods is challenging because of the exorbitant calculation times required by current methods for contemporary data sizes, the difficulty in correctly modeling the rate heterogeneity in highly diverse taxonomic groups, and the lack of reliable clock calibrations and their uncertainty distributions for most groups of species. Here, we present a method that estimates relative times of divergences for all branching points (nodes. Nucleotide sequence of gene encoding for viral coat protein of red spotted grouper nervous necrosis virus (RGNNV) was used as an outgroup for the comparison between the genus. The construction of phylogenetic trees was conducted by matching the nucleotides sequence of the isolates from Sarawak (A, B, C), Perak (TB, TC, TE, TL) and Perlis (TiTT 125 – 127) with four nucleotide sequences of segment 3 from other countries i.e., Thailand (Tattiyapong, Dachavichitlead and Surachetpong 2017), India (Behera et al., 2018), Israel (Eyngor et al., 2014) and Ecuador (Bacharach et al., 2016).

The evolutionary history and analysis were inferred using the Maximum Likelihood method based on the Tamura-Nei model (K Tamura and Nei 1993). The tree with the highest log likelihood (-1017.2769) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbou-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Branch lengths are expressed in terms of the number of substitutions per site, and the tree is depicted to scale. 15 nucleotide sequences were subject to the analysis. There were no positions left that had blanks or missing data. The final dataset included 241 positions in total. Software called MEGA 7 was used to assess evolutionary data (Kumar, Stecher, and Tamura 2016).

The evolutionary history and analysis were somewhat inferred with an approach of utilizing the Maximum Likelihood method based on the Tamura-Nei model (K Tamura and Nei 1993). The tree that presents the highest log likelihood (-1017.2769) is shown. Initial tree(s) for the heuristic search were achieved automatically by applying Neighbor-Join and BionNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) method, which then subsequently led to choosing topology with superior log likelihood value.

Results and Discussion

Virus inoculation

Inoculation of supernatant from positive isolate of TiLV in E-11 cell lines resulted in the CPE after three to five days post inoculation (pi) (Fig 1). There are 33 out of 62 samples (53.2%) inoculated into cell culture were positive for CPE. Figure 1 (B), the cell line shows visible cytopathic effect (CPE) at day 3 post infection with cytoplasmic vacuoles and plaque formation compared with control, Fig 1 (A). The cell monolayer disintegrates after nine to ten days of inoculation, suggestive the viability of the virus, compared with control cells which are still intact.

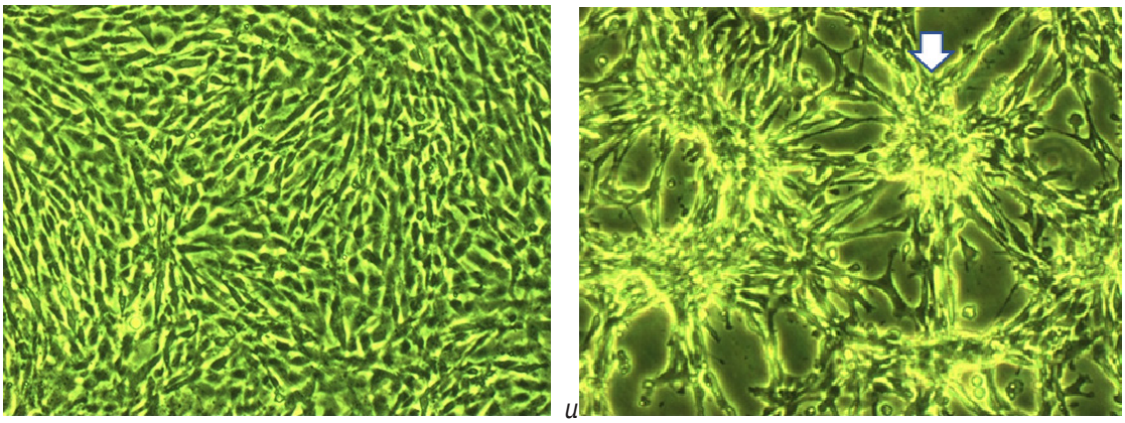

Figure 1. Uninfected E-11 cells, (A) and (B) cytopathic effect (CPE) formation in E-11 cells infected with TiLV at day 3 pi (white arrow).

RT-PCR and sequencing analysis

All positive samples gave a single amplicon of 245bp indicating light infection of TiLV (Fig 2). The sequencing results for segment 3 of TiLV were aligned with the database in GenBank using NCBI nucleotide BLAST (blastn), showed that all TiLV isolated from respective localities had closest kinship with TiLV isolate from Thailand (accession number: KY381578.1) i.e., 98% similarity (Fig. 3). However, this sequence is too small for the author to draw much out of it.

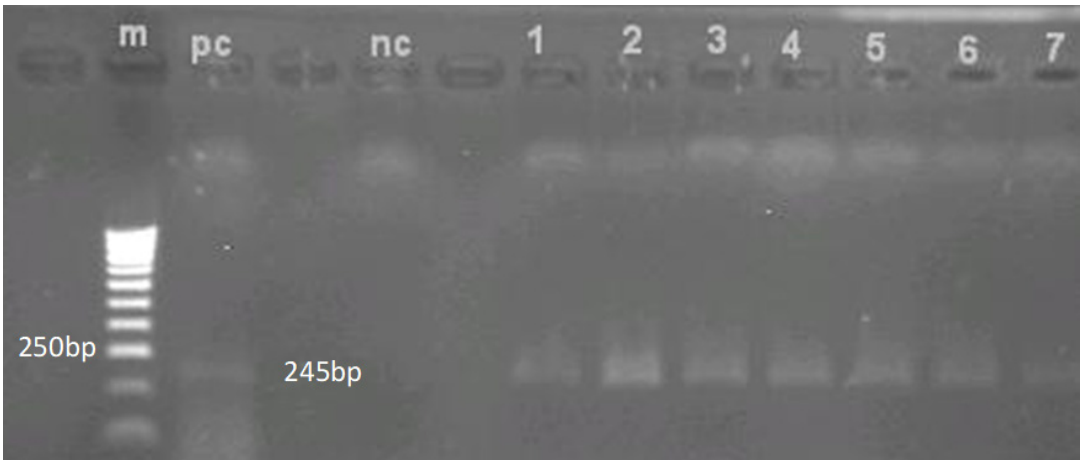

Figure 2. Agarose gel image of TiLV detection using semi-nested RT-PCR assay with RNA template from Sarawak isolates. All samples produced 245 bp amplicon. Lane M = DNA marker (100bp DNA ladder, Hyperladder MyTaq, Korea); pc = positive control; nc = negative control, lane 1-7 TiLV infected tilapia.

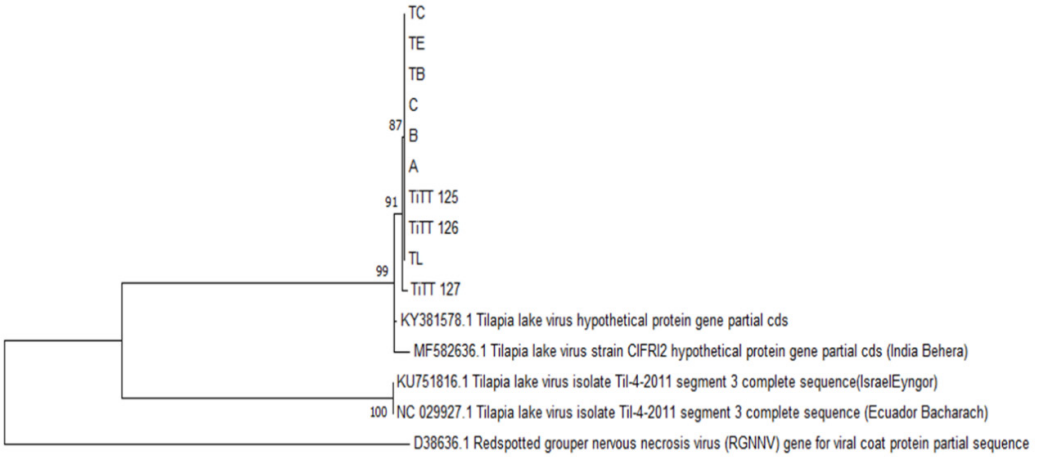

Figure 3. Phylogenetic tree showing the relationship between TiLV strain from Sarawak (A, B, C), Perak (TB, TC, TE, TL) and Perlis (TiTT 125 – 127) aligned with other published segment 3 of TiLV sequences deposited in GenBank. The tree was determined using neighbour-joining methods.

Among the 10 segments of TiLV, only Segment 1 has been extensively studied. Based on the phylogenetic tree, TiLV sequences separated into three clades namely Israeli – 2011, Israeli – 2012 and Thai clade which closely related to monophyletic Israeli – 2012 clade (Taengphu et al., 2020). Nucleotide sequences similarities of the viral isolates obtained from different countries shows 97.2% – 99% similarities between the Ecuador and Israel strains, 95.38% – 95.68% for Thailand – Peru strains, 96.32% – 97.71% in Thailand – Israel strain and 96.89% – 97.13% in Israel – Peru strains based on the genome sequence analysis (Pulido et al., 2019). Latest genomic study of TiLV strains reveal that the Ecuador strain has 95.77% similarities with TiLV strains isolated from Thailand and 97.24% similarities with strain from Peru (Aich et al., 2022). Isolates from Peru and Thailand was identified to be closely related with Israel strain although the genetic variation from both of the country shows genetic distant between themselves that suggest the infection of TilV in Thailand and Peru might have caused by the strains from Israel (Aich et al., 2022). Furthermore, TiLV from Thailand shares high sequences similarity with TiLV from Israel, Chinese Taipei, Ecuador, Egypt, India, Indonesia and Philippines (Jansen, Dong and Mohan 2019). Recently, phylogenetic analysis of the TiLV from United States showed that their isolates are most closely related to Thailand TiLV strains (Ahasan et al., 2020)we present the complete coding sequences of two tilapia lake virus (TiLV. Phylogenetic analysis of TiLV isolated from fish in Lake Victoria also showed homology with outbreaks in Israel and Thailand (Mugimba et al., 2018)and of the 442 samples examined from 191 fish, 28 were positive for TiLV by PCR . In terms of tissue distribution, the head kidney (7.69%, N = 65. This suggesting that the virus spreads across continents and tilapia trading around the world came from the same source (FAO, 2016).

Phylogenetic analysis is able to track how pathogens have dispersed across the globe, and inferences about the movement of TiLV have been made based on phylogenetic analysis of short sequences from a single segment (Nicholson et al., 2017; Surachetpong et al., 2017). Whether this is appropriate for TiLV, with its segmented, negative-sense RNA genome that may be prone to re-assortment, does not appear to have been tested, though previous studies with limited sequence data noted an absence of re-assortment in this virus (Nicholson et al., 2017; Pulido et al., 2019). However, recent study conducted by Chaput et al., (2020) using TruSeq RNA library preparation following the standard manufacturer's protocol (Illumina Inc., San Diego CA, USA) which further analysed

using Quartet Tree Analysis (Suzuki, 2010), shows the nucleotide sequence of TiLV can and do undergo reassortment process which may contribute to different evolutionary records outcomes and inaccurate estimation of the virus evolutionary history based on the multiple region genomic analysis. Recent study showed that phylogeny of TiLV isolates depends on the segment sequence; in particular, segments 5 and 6 appear to have undergone a relatively recent re-assortment event (Chaput et al., 2020). The findings illustrate the need to exercise caution when using phylogenetic analysis to infer geographic origin and track the movement of TiLV.

Conclusion

Future research should look at and demonstrate the capacity of local isolates to inflict diseases and virulence in susceptible fish, since these findings were predicated on nucleic acid detection by PCR. The results present in this article is preliminary, and only focus on a short frame of one gene of TiLV i.e., segment 3. More studies need to be done, and we recommend using whole genomes wherever possible or at least segment 5 and 6 which appear to have undergone a re-assortment.

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Indian Mackerel (*Rastrelliger kanagurta*): Stock Status and Resources in Northern West Coast of Peninsular Malaysia

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Abstract: Indian mackerel (*Rastrelliger kanagurta*) is one of the common and economically important pelagic fish in Malaysia. This study was conducted from January 2017 to December 2018. Samples were collected monthly from the commercial purse seines in Kuala Perlis, Perlis landing site. The objective of this study was to assess the resources and stock status of Indian mackerel in the northern area of the West Coast of Peninsular Malaysia. The catches and Catch Per Unit Effort (CPUE) trend in the West Coast Peninsular Malaysia (WCPM) showed fluctuation from 2008 to 2018. The spawning season for the Indian mackerel in WCPM were estimated to be in December to March. The maximum spawning activity occurred in January with the male and female GSI at 4.3 and 3.7 respectively. In WCPM, the highest landing of Indian mackerel comes from purse seines and trawls net. The analysis from Kobe plot showed the stock status of Indian mackerel in WCPM was in the green zone implying it is still safe to be harvested.

Keywords: Indian mackerel, spawning season, stock status, West Coast of Peninsular Malaysia

Abstrak: Ikan kembong (*Rastrelliger kanagurta*) merupakan spesies yang biasa di jumpai dalam kumpulan ikan pelagik di Malaysia. Kajian status stok dan sumber ikan kembong telah dijalankan dari Januari 2017 hingga Disember 2018. Sampel ikan kembong diperolehi dari tangkapan pukat jerut di jeti pendaratan ikan Kuala Perlis. Objektif kajian ini adalah untuk mentaksir status stok dan sumber ikan kembong di bahagian utara pantai barat Semenanjung Malaysia. Tangkapan per unit usaha (CPUE) di pantai barat menunjukkan corak turun naik dari tahun 2008 hingga 2018. Musim pembiakan ikan kembong di kawasan pantai barat dijangka berlaku pada bulan Disember - Mac. Aktiviti pembiakan yang tinggi dapat dilihat pada bulan Januari dengan nilai gonadosomatik bagi jantan dan betina masing-masing sebanyak 4.3 dan 3.7. Analisis Plot Kobe pula menunjukkan status stok ikan kembong masih berada di zon hijau yang memberi gambaran bahawa spesies ini masih boleh di eksploitasi secara mapan.

Introduction

The Indian mackerel, (*Rastrelliger kanagurta*) is one of the economically important epipelagic fish species in Malaysia. An epipelagic fish inhabits the epipelagic zone *i.e.,* water from the surface of the sea down to 200 metres (Lønborg et al., 2016) and also referred to as the surface waters or the photic zone. The Indian mackerel belongs to the Scombrid family of the Perciformes Order (Froese and Pauly, 2021). This species is commonly distributed across the Indo-West Pacific from South Africa, the Seychelles and the Red Sea, east of Indonesia and Northern Melanesia, Micronesia, Samoa, China and Ryukyu Islands of Southern Japan (Akib et al., 2015).

The Indian mackerel is abundant across the waters off north-west and east of Peninsular Malaysia, Sabah and Sarawak (Mansor 1997; Chee 1999). They feed mainly on zooplankton, phytoplankton and algae (Hulkoti et al*.,* 2013). It is a highly migratory species with a streamlined fusiform body that make them fast swimmers and habitually found in big schools. In Malaysia, the Indian mackerel contributed about 4.87 % of the total marine fish landings in 2021 (Department of Fisheries Malaysia, 2021). It was also the third highest pelagic species landed after the short mackerel and scads. Figure 1 shows the landing of the important pelagic species according to the fishing areas in 2021.

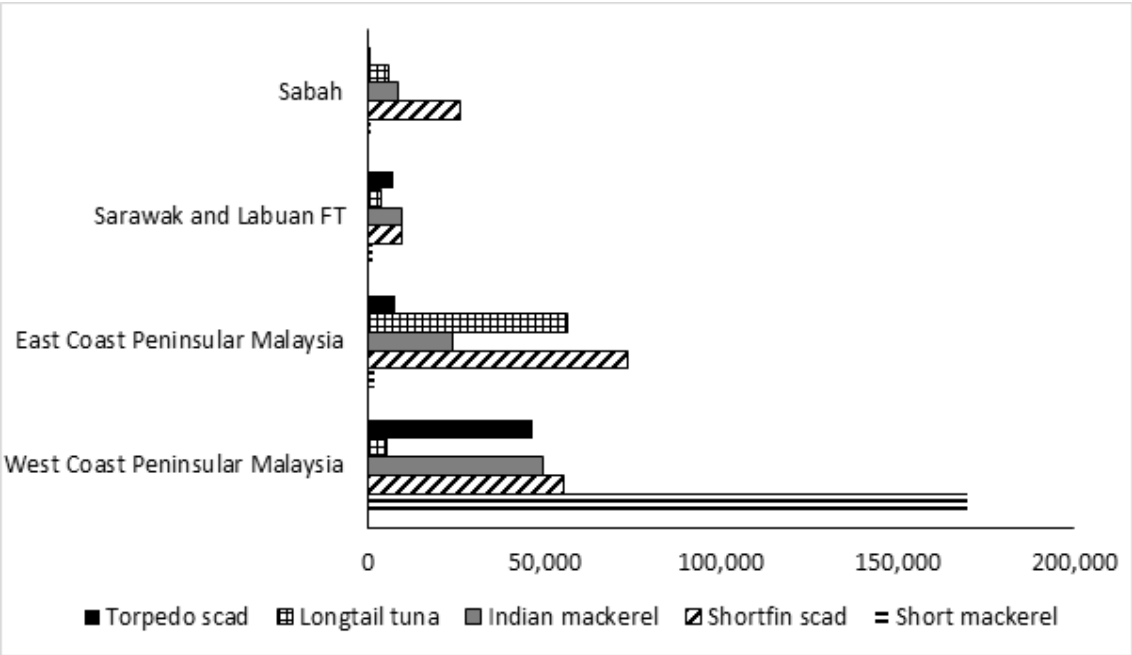


Figure 1. The landings of important pelagic species (tonnes) by fishing areas in 2021

The fishing waters of Malaysia can be mainly divided into four zones, West Coast of Peninsular Malaysia (WCPM), East Coast of Peninsular Malaysia (ECPM), Sabah and Sarawak. The WCPM covers the Strait of Malacca that embraces the north of the Andaman Sea and Indian Ocean. The main fishing gears commonly used to catch the Indian mackerel are purse seine and trawl net. In 2021, about 34.82% out of 40,959.95 tonnes of Indian mackerel landed in the country was contributed by purse seine while 26.34% from trawl net (Department of Fisheries Malaysia, 2021). There is possible usage of other tools by the purse seiners such as fish aggregating devices (FADs) or light to lure the fish into the net.

There are various methods used for stock assessment depending on the types of data, knowledge used and degree of age-structured population dynamics in the models (Cadrin and Collas, 2015). One of the methods, Kobe or phase plot analysis had been utilized to evaluate the status of tuna stock based on the fishing mortality (F) and biomass (B) associated with Maximum Sustainable Yield (MSY; *i.e.,* FMSY and BMSY) (Maunder and Aires-de-Silva, 2011). This method was introduced by the Tuna Regional Fisheries Management Organization (RFMOs), held in Kobe, Japan in January 2007.

The aim of fishery management is to ensure sustainability in harvesting fish stocks and to avoid depletion of the resources. A successful or accurate stock assessment is very important so that fishery management for certain species can be developed depending on the estimation on quota and allowable catch regulation. This is applicable to all fish including the Indian mackerel. The resources should be managed at the optimum level since it is an important commercial fish and among 10 most preferred fish in Malaysia (Nurul Izzah et al., 2016).

This study utilizes the Kobe plot analysis to determine the current stock status of the Indian mackerel in the WCPM. The information provided through the biology and stock assessment in this study may be used by the fisheries managers to formulate the best strategy to ensure the sustainable management of this valuable resource in Malaysia.

Materials and Methods

Biological data were collected from from 2017-2018 for gonad maturity study. Samples of the Indian mackerel were collected monthly from the commercial purse seiners at Kuala Perlis, West Coast of Peninsular Malaysia landing site. In total, 3,318 Indian mackerel samples were collected and measured for gonadosomatic index estimation (GSI). The GSI is a metric that represents the relative weight of the gonad to the fish weight. Next, the length and weight of the fish were measured by using a ruler with 1 mm accuracy and an analytical scale with an accuracy of 0.01 gram. Gonads were observed and removed, then weighted on the analytical scale of 0.01 gram for GSI purpose.

Secondary data was obtained from the Department of Fisheries, Malaysia to elucidate the catch and landing trend of the Indian mackerel in Malaysia. For the stock assessment study of the Indian mackerel, the Kobe plot were used to analyse the data. There are two main visualization tools used in the Kobe Framework; a phase plot and strategy matrix. The phase plot visualized the current stock status and exploitation rate according to the targeting points such as BMSY and FMSY of the Indian mackerel. On the other hand, the strategy matrix represents the probability of catch according to the management objective such as the Total Allowable Catch (TAC). This will be translated according to colours stated such as green (safe zone), yellow (recovery), and red (overfished).

Results and Discussion

Gonad maturation study

The spawning season for the Indian mackerel in WCPM were estimated to be in December to March based on the highest value of GSI for both sexes (value ranged between 2.7 - 4.3) (Figure 2). The maximum spawning activity occurred in January with the male and female GSI at 4.3 and 3.7 respectively. The size at first maturity was estimated to be 19.6 cm for male and 20.4 cm for female. The fecundity of fish ranged between 25,144 – 151,256 eggs.

Seasonal variations in the values of GSI were reported by different researchers for different areas. Rahman and Hafzath (2012) stated the spawning season of Indian mackerel in Kuantan coastal water falls within the period between end of January and end of May. Furthermore, others studies stated that there are two spawning seasons of *Rastrelliger kanagurta* in Western waters of Aceh, the first from January to March and then from August to October (Arrafi et al., 2016). Spawning season for present study coincides with several studies from adjacent waters (Table 1).

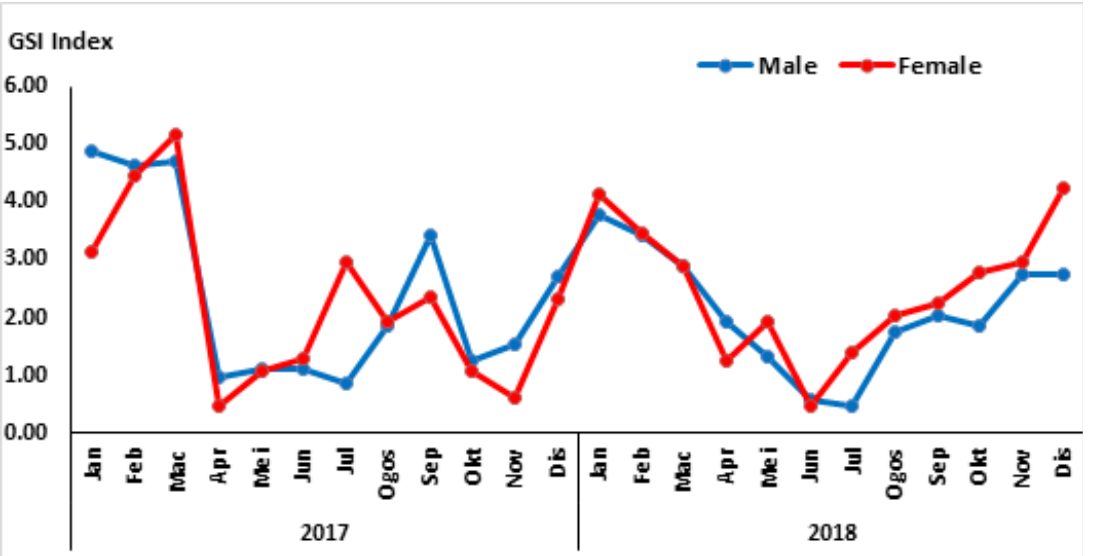


Figure 2 Average gonado-somatic index of the Indian mackerel in the West Coast Peninsular Malaysia, 2017-2018 .

Table 1Spawning season of Indian mackerel from adjacent waters

Spawning season	Study area	References
December – January	Kakinada, India	Abdussamad et al., (2006)
June – August	South China Sea	Suwarso et al., (2008)
January – May	Kuantan coastal, Malaysia	Rahman and Hafzath (2012)
January – March August – October	Western waters of Aceh, Indonesia	Arrafi et al., (2016)
October – November	Kwandang waters, north Gorontalo, Indonesia	Faizah et al., (2017)
April and August	West Bengal, India	Das et al., (2016)
December-March	West coast Peninsular Malaysia	Present study

Catch and landing trend

Figure 3 illustrates catch trends for the Indian mackerel in WCPM from 2008 to 2018. In general, the catch trend shows a gradual increase in catch from 2008 (20,540 tonnes) to 2012 (40,229 tonnes) before dropping slightly in 2013 with catches at 29,000 tonnes with an ascending trend again in 2014 with the highest catch recorded at 44,400 tonnes. The catch however gradually decreased again from 2015 to 2017 and raised again in 2018 at 27,738 tonnes. The catch per unit effort (CPUE) also showed similar trends as the catch.

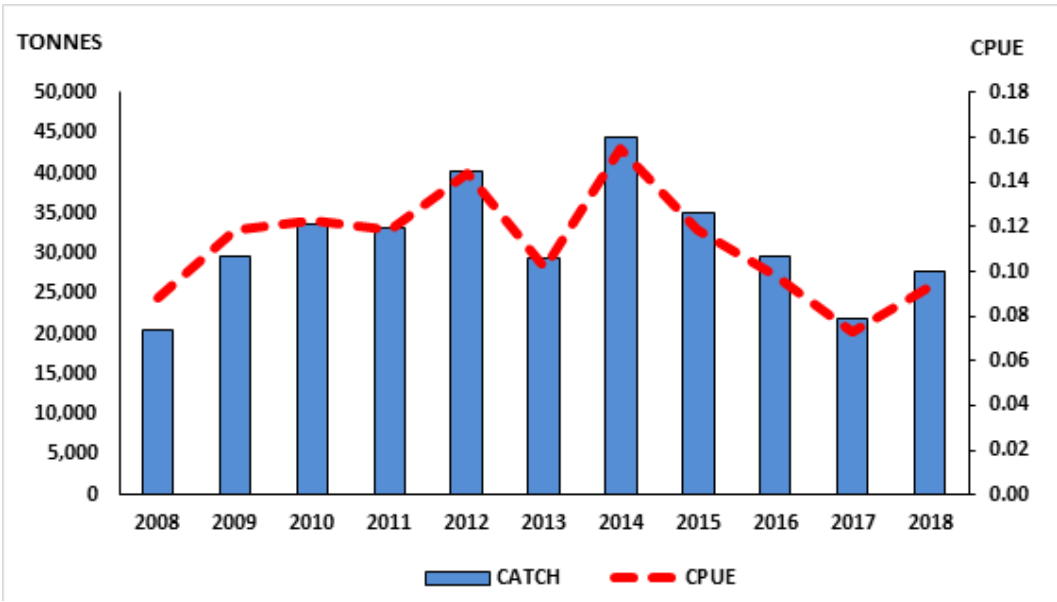


Figure 3. Catch and CPUE of the Indian mackerel in the West Coast of Peninsular Malaysia, 2008 to 2018

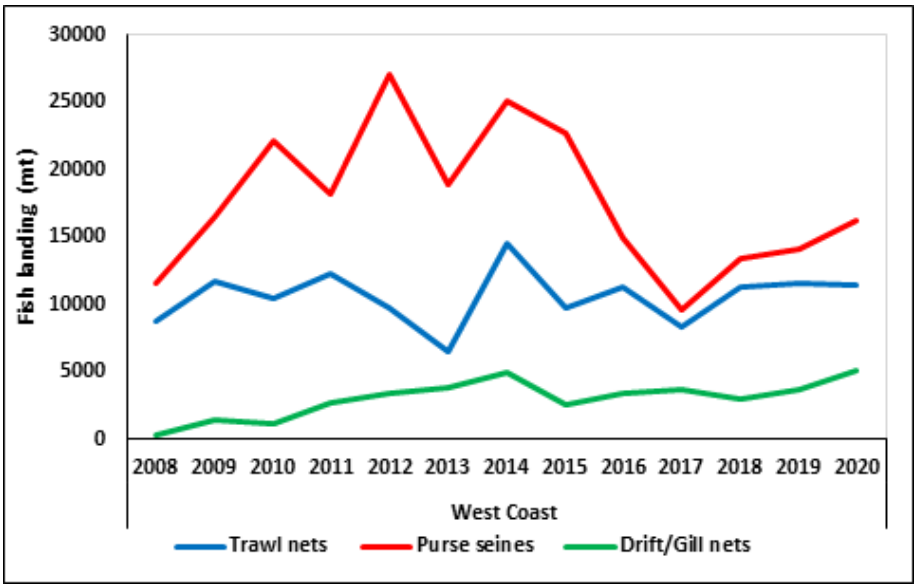


Figure 5. Landing by gears of Indian mackerel in West Coast Peninsular Malaysia, 2008 to 2020

In West Coast of Peninsular Malaysia, the highest landing of Indian mackerel from 2008 to 2020 was by purse seines (214,124 mt) then followed by trawl nets (126,184 mt) and drift/gill nets (33,845 mt) (figure 5). The highest landing of Indian mackerel by purse seines was in 2012. Figure 7 showed catch composition percentage of pelagic fish by purse seines in 2008 to 2020. In West Coast of Peninsular Malaysia, the Indian mackerel (15%) was among the highest catch percentage after short mackerel (31%) and Scads (23%) which are also deemed as '*Ikan Rakyat*". Thus, it showed that Indian mackerel were one of the targeted species by major fishing gears in the WCPM as it serves as cheap and reliable source of protein for Malaysian.

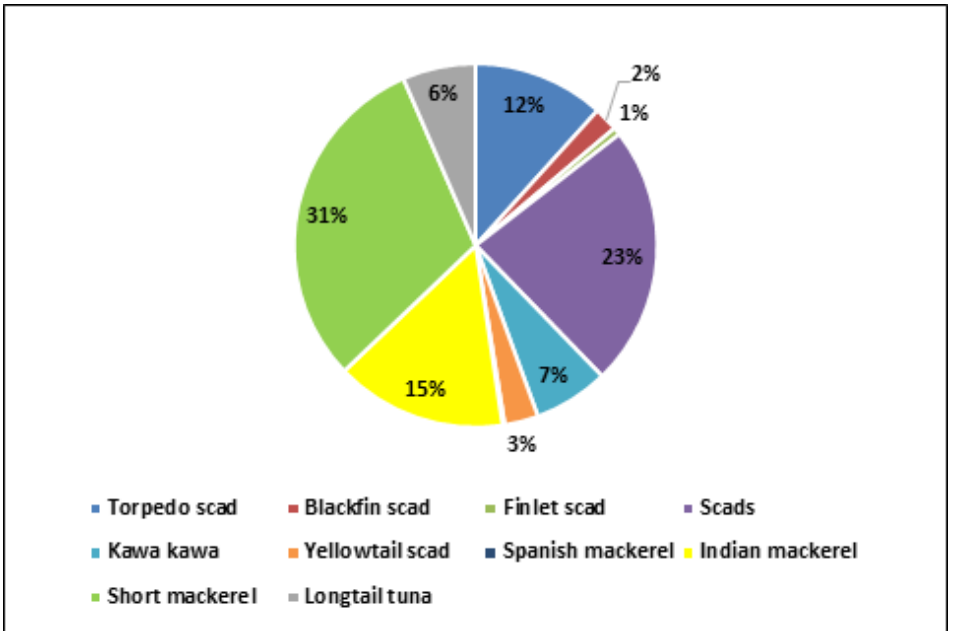


Figure 7. Catch composition of pelagic fish by purse seines in West Coast Peninsular Malaysia from 2008 to 2020.

Based from the stock and risk assessment of the Indian mackerel using Kobe plot analysis showed that the status of this species in WCPM was in the green zone implying that the stock was still safe to be harvested (Figure 5). The current catch level was 26,405 tonnes whereas the risk of exceeding the TBmsy and Fmsy was 30 %. Thus, the total catch of the Indian mackerel in the WCPM could be increase by additional 20 % (31,686 tonnes) while reserving 10 % as a buffer to overexploitation to ensure that the stock can be sustained for the next 10 years.

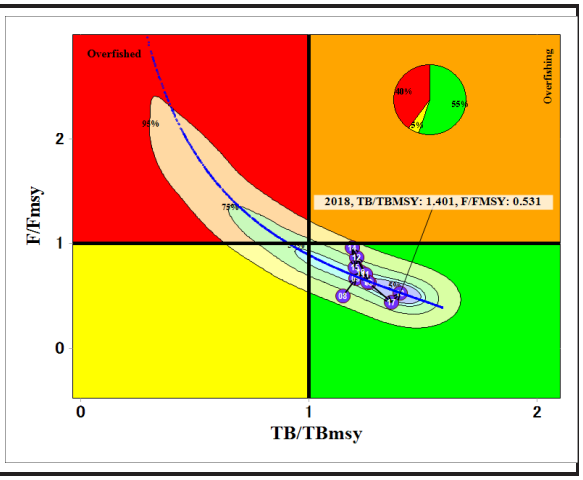
The Indian mackerel (West Coast Peninsular Malaysia)	
Red zone (40%) and Green zone (55%) Maximum Sustainable Yield (MSY) : 39,608 tonnes Current catch : 26,405 tonnes* TB/TBmsy : 1.401 F/Fmsy : 0.531 Advice: TAC > 5,000 t (increase 20% from *)	

Figure 5. Stock status (2018) and management advices (Total Allowable Catch/TAC) based on stock assessment by Kobe Plot Analysis

Conclusion

The stock status of the Indian mackerel in Malaysia as of 2018, being in the green zone for West Coast Peninsular Malaysia implies that the stock is still safe to be harvested. Hence, based on the current catch level, a proper management should be taken into consideration to ensure that the stock of the Indian mackerel lies within its safe zone or can be sustained for the next decade.

The collaboration with neighbouring countries such as Thailand and Indonesia should also be considered due to the migratory-epipelagic nature of this species. Through the establishment of Technical Working Group, activities such as information exchange can be developed. Skills development through capacity building or training program can be developed and upgraded. A cooperation between scientist and managers also could be facilitated so the development of information, education and communication programs on sustainable use of resources can be supported thus management measures to control fishing effort and capacity at national or even regional level can be established.

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INSTRUCTION TO AUTHORS

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Manuscript should be prepared in Microsoft Word. The paper must be typed with a double spacing throughout, including references, tables, footnotes, figures legends, etc on A4 size paper leaving margins of 25 mm minimum. Line numbers should be insert for review purposes. Headings should be centered, upper case in bold, size 14. Sub-headings should be lower case, centered and in bold. Sub-sub-headings should be in *italics* at left margin. The font used throughout your document should be in Times New Roman, 12-point font size.

Title Page:

a) A concise and informative title unobscured by taxonomic detail.
b) Name of author(s) should be in full, capital letters, font size 12.
c) Address of institution(s) where the work was done should be written in full, italic, font size 11.
d) Corresponding author email - mark with *, *italic.*

Abstract and Keywords

Abstract: Hanging indent, *italic,* , fobldw by text
Two abstracts required: English and Bahasa Malaysia version
Provide: i. No more than 200 words summarizing the main points of the paper
ii. Up to six keywords or phrases

Introduction

It should include key references to appropriate works and up-to-date primary literature. The rationale of the research undertaken should be explained. The introduction should clearly state the aims and objectives of the paper.

Materials and Methods

Materials and Methods should be described in sufficient detail to allow the work to be repeated. Specify and describe the study site and test animals where appropriate. Sub headings are used to itemize the main parts. Materials and methods should be written in the past tense either in active of passive voice. In this section, study dates, number of subjects, groups, evaluation criteria, exclusion criteria and statistical methods should be described sequentially. The origin of materials and/or suppliers of equipment should be named if necessary.

Results and Discussion

The sections may be separated, though authors may find it's easier to combine them. Use tables or graphs as appropriate but do not repeat information in the text. The reproducibility of the findings must be clearly stated, the number of times the experiment was conducted, the number of replicate samples, etc., should be stated. Statistical analysis of results must specify the procedure being used with a reference given. If results are given as a percentage of a control value, the 100% should be given. Discussion should provide the explanation and interpretation of results or findings by comparing with the prior studies. It should bring out those essential points of the work, the implications and practical significance of the findings, their limitation and relevance to previous studies. It should not be a

recapitulation of the results.

References

The references follow APA style. In the text, references should be cited as: Smith (1993) or (Smith, 1993). Two authors as: Smith and Brown (1993) or (Smith and Brown, 1993). Three or more authors as: Smith et al. (1993) or (Smith et al., 1993). A series of references should be appearing in chronological order, e.g., White and Black 1991; Black and White 1992. References to papers by the same authors in the same year are distinguished by letter a, b, etc. (e.g., 1989a, or 1991a, b). Publications having no obvious authors are cited as Anon. (1990) in the text and bibliography. References with 4 and more authors should be written as: Abbasi, A. A., Paparidis, Z., Malik, S., Goode, D. K., et al. (2007). References to grey literature such as in-house reports, contract reports and non-referred papers are not appropriate and should be avoided. At the end of papers, References are listed in alphabetical order by the first word in the reference (usually the author's last name). References with three or more authors should be placed in chronological order after considering of the names of the first and second authors. The author must ensure that references cited in the text agree with those listed in the bibliography. Some sample reference styles follow:

a) Citation for Journals
i. In print
Sequence of citation: author's name, initials (for each author) (year of publication).
Full title of paper. *Name of journal* (abbreviated in accordance with the Bibliographic Guide), volume (issue nu), first page - last page.

Example: Saha, B. C. and Zeikus, J. G. (1989). Improve method for preparing high maltose conversion syrup. *Biotechnology and Bioengineering*, 34, 229-303.

Example: Debnath, P. P., Delamare-Deboutteville, J., Jansen, M. D., Phiwsaiya, K., et.al (2020). Two-year surveillance of tilapia lake virus (TiLV) reveals its wide circulation in tilapia farms and hatcheries from multiple districts of Bangladesh. *J Fish Dis.* 43(11),1381-1389.

ii. Online
These references are formatted the same way as the print versions, except the DOI or URL is included at the end. If the article has a corresponding DOI number, use it instead of the URL. No URL? Use the homepage of the journal's website for the URL.

Author's Last name, F. M. (Year published). Title of article. *Title of Journal*, volume number (issue number), page range. https://doi.org/10.xxxx/xxxxxx OR URL

Example:
Spreer, P., and Rauschnabel, P. A. (2016). Selling with technology: Understanding the resistance to mobile sales assistant use in retailing. *Journal of Personal Selling and Sales Management*, 36(3), 240-263. https://doi.org/10.1080/08853134.2016.1208100

b) Citation for Books

i. Sequence of citation for print books: author's or editor's name, initial, (year of publication), *book title.* Publisher, and place of publication.
Capitalize the first letter of the first word of the title and any subtitles, as well as the first letter of any proper nouns. The full title of the book, including any subtitles, should be stated and italicized.

Example: Primrose, G. B. (1987). *Modern Biotechnology*. Blackwell Scientific, Oxford.

ii. Citations for Edited Books
Most edited books state on the cover or title page that they are edited by an author or multiple authors. The format is the same as a print book, except the editor's name is in the author's position. Include a parenthesis afterwards with the abbreviation (Ed.) for an edited book by one author or (Eds.) for an edited book with two or more authors.

Editor, F. M. (Ed.). (Year published). *Title of edited book*. Publisher.

Example:
a) Primrose, G.B. (Ed.). (1987). *Modern Biotechnology*. Blackwell Scientific, Oxford.

b) Gudding, R., Lillehaug, A. and Evensen, O. (Eds.). (2014). *Fish Vaccination*, John Wiley & Sons Ltd., UK.

iii. Citations for Chapters in Edited Books
Some edited books contain chapters written by various authors. Use the format below to cite an author's individual chapter in an edited book.

Chapter author's Last name, F. M. (Year published). Title of chapter. In F. M. Last name of Editor (Ed.), *Title of book* (p. x or pp. x-x). Publisher.

The title of the chapter is not italicized, while the title of the book is. The chapter author's name is reversed at the beginning of the reference, but the editor's name is written in standard order.

Example:
a) Longacre, W. A., and Ayres, J. E. (1968). Archeological lessons from an Apache wickiup. In S. R. Binford and L. R. Binford (Eds.), *Archeology in cultural systems* (pp. 151-160). Blackwell, Oxford, UK.

In the above example, Longacre and Ayers are the authors of the individual chapter and Binford and Binford are the editors of the entire book.

b) Gudding, R. (2014). Vaccination as a preventive measure. In R. Gudding, , A. Lillehaug, and O. Evensen, (Eds.), *Fish Vaccination* (pp. 12-21). John Wiley & Sons Ltd, West Sussex, UK.

c) Citations for conference/proceedings

Conference proceedings published as a whole book follow the same reference format as whole: i) journal, ii) edited books or iii) book chapter

Example:

i. Duckworth, A. L., Quirk, A., Gallop, R., Hoyle, R. H., Kelly, D. R., and Matthews, M. D. (2019). Cognitive and noncognitive predictors of success. *Proceedings of the National Academy of Sciences*, *USA*, *116*(47), 23499–23504. https://doi.org/10.1073/pnas.1910510116

ii. Kushilevitz, E., and Malkin, T. (Eds.). (2016). *Lecture notes in computer science: Vol. 9562. Theory of cryptography*. Springer. https://doi.org/10.1007/978-3-662-49096-9

iii. Benedel, A. L., Jourdan, L. and Biernacki, C. (2019). Probability estimation by an adapted genetic algorithm in web insurance. In R. Battiti, M. Brunato, I. Kotsireas & P. Pardalos (Eds.), *Lecture notes in computer science*: Vol. 11353. Learning and intelligent optimization (pp. 225-240), Springer. https://doi.org/10.1007/978-3-030-05348-2 21

a) Citations for Newspapers found Online
Use this structure when referencing a newspaper article found on a website or database:

Author's Last name, F. M. (Year, Month Day of Publication). Title of article. *Title of Newspaper.* URL of newspaper's homepage

Example:
Rosenberg, G. (1997, March 31). Electronic discovery proves an effective legal weapon. *The New York Times.* http://www.nytimes.com

b) Composite works of serials:
Sequence of citation: author's name, initials, year of publication, publisher, place of publication, first and last page no.

Example:
Guilbot, A. and Marcier, C. 1985. Starch. *In:* Aspinall, G.O. (ed). The polysaccharides, Academic Press, New York, pp. 209-283.

c) Full publication details must be given for any citation that does not fit into any of the above categories such as unpublished in-house reports, contract reports, etc.

Acknowledgement

Brief of appreciation to whom it is due.

Table

Plain Tables should be used for data which cannot be described in the text. Type each table double spaced and position in the manuscript. Table number and caption should be positioned at the top. Explanatory footnotes in lower case letters should be concise to enable them to stand independent of the main text. Tables are numbered with Arabic numerals.

Figures

Figures should be selected to illustrate points which cannot be easily made in the text. They are numbered with Arabic numerals. Graphs, photos and diagrams with caption should be positioned in the manuscript. Diagrams must be drawn and lettered in black ink on good quality white paper for camera-ready use. Lettering should be parallel to the axes. Photocopies, hand-drawn diagrams and typewritten labels are not acceptable. Scale marks on graphs should be within the axes. Graphs should avoid as far as possible large areas of unused space.

Photographs should be will-contrasted black and white prints. For photomicrographs, the magnification should be given a scale (or marker) bar on each photograph and the length of this represents given in the legend. Photographs of the original material should be submitted for the reviewer's scrutiny and the purpose of printing.

Units, Abbreviations and Nomenclature

Use only recommended SI Units. Use superscripts presentation (mg mL-1). Below are few examples of abbreviations of the most commonly used SI units:

Base quantity	Name	Abbreviation
Length	Meter	m
Mass	Kilogram	kg
Time	Second	s
Time	Minute	min
Electric current	Ampere	A
Area	square meter	m2
Volume	cubic meter	m3
Frequency	Hertz	Hz

The correct Latin names of organisms must be used on first mention in the text. A widely recognized and designated common name should be used for subsequent mention.

References

American Psychological Association. (2020). *Publication manual of the American Psychological Association* (7th ed.). https://doi.org/101037/0000165-000

Paper Title: A concise and informative title unobscured by taxonomic detail

FULL NAME ALL AUTHORS: AUTHOR1,2, AUTHOR3, AUTHOR3, AUTHOR1, AUTHOR4,*

1 Institution with complete current address, including post code

2 Institution with complete current address, including post code

3 Institution with complete current address, including post code

4 Institution with complete current address, including post code

*Corresponding author: author@institution.domain

Abstract: A concise and factual abstract is required and must be written in English (maximum length of 250 words). The abstract should state briefly the purpose of the research, the methods used, the principal results and major conclusions. Please try to keep each sentence as specific as possible and avoid such general statements as "The fisheries status are discussed'. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, they must be cited in full, without reference to the reference list. Any abbreviations should be avoided, but if needed they must be defined at their mentioned in the abstract.

Keywords: Provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abstrak: Abstrak di dalam Bahasa Malaysia mestilah mempunyai makna dan terjemahan maksud yang sama seperti dalam abstrak Bahasa Inggeris. Penggunaan 'google translate' dibenarkan dengan syarat penulis memeriksa kembali setiap patah perkataan dan membuat pembetulan mengikut tatabahasa yang betul.

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Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described. The correct Latin names

of organisms must be used on first mention in the text. A widely recognized and designated common name should be used for subsequent mention.

Methods

Sub headings are used to itemize the main parts. Materials and methods should be written in the past tense either in active of passive voice. In this section, study dates, number of subjects, groups, evaluation criteria, exclusion criteria and statistical methods should be described sequentially. The origin of materials and/or suppliers of equipment should be named if necessary.

Sub-section

Third level section should be italic. We do not encourage additional sub-levels after the third level. Please try to make your paper concise and clear.

Units, Abbreviations and Nomenclature

Use only recommended SI Units. Use superscripts presentation (e.g: mg mL-1) and common abbreviations such as 'm' for meter, 'kg' for kilogram, 'min' for minute and so on.

Results and Discussion

Results should be clear and concise. The discussion should explore the significance of the results of the work. Avoid extensive citations and discussion of published literature. If appropriate, Results can be written in a separate section from Discussion. This especially if the Discussion is extensive and includes all the Results of the study.

Table

Please submit tables as editable text and not as images. Tables use double space and 12pt Times New Roman fonts.

Table 1. Use Times New Roman 12 font

Component	Content (%, w/w)
Protein	44.9 ± 0.37
Carbohydrate	22.3 ± 0.94
Water content	13.7 ± 0.02
Ash	6.1 ± 0.19

Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

Figures

Please embed the figures in the text with minimum resolution of 300 dpi. Separate figure files in JPEG or PNG formats can be supplied if it feels necessary. Ensure that each figure has a caption. A caption should comprise a brief title (not on the figure itself) and a description of the figure. Keep text in the figure themselves to a minimum but explain all symbols and abbreviations used.

Figure 1. Left: Trap one funnel (1F)

Graphs

Graphs must be supplied in figure formats. The fonts of the graph must be clear and readable. Black and white graphs are preferred.

Citation

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list, they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication. All manuscripts should be formatted using the American Association style (APA). You can download the APA style for reference manager (Mendeley, Zotero, etc.) from the trusted website.

Text

All citations in the text should refer to:

• Single author: the author's name (without initials, unless there is ambiguity) and the year of publication (Ratledge, 2002); OR Ratledge (2002).
• Two authors: both authors' names and the year of publication (Triyaswati & Ilmi, 2020);
• Three or more authors: first author's name followed by 'et al,' and the year of publication (Papanikolaou et al., 2011).

Reference list

References should be arranged first alphabetically and then further sorted chronologically. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of Results and Discussion section.

Author contribution (Optional)

Please list the contribution of each author here, e.g.: M.I. designed the research and supervised all the process, L.A. collected and analyzed the data and wrote the manuscript.

Acknowledgments

List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proofreading the article, etc.).

Conflict of Interest (Optional)

Please state any conflict of interest regarding the research or the research funding.

References

Debnath, P.P., Delamare-Deboutteville, J., Jansen, M.D., Phiwsaiya, K. et al. (2020). Two-year surveillance of tilapia lake virus (TiLV) reveals its wide circulation in tilapia farms and hatcheries from multiple districts of Bangladesh. *Journal Fish Disease,* 43(11),1381-1389.

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CONTENTS

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